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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Tyrosine phosphorylation of p27Kip1

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Tyrosine phosphorylation of p27Kip1

Field of the invention

The present invention is directed to tyrosine phosphorylated forms of p27Kip1, fragments of these forms and antibodies thereto. The invention is further related to non-phosphorylable mutant forms of p27Kip1 or fragments thereof. Further embodiments are diagnostic or therapeutic uses of the disclosed compounds, in particular uses in diagnostics and therapy of hyperproliferative diseases.

Background of the invention

Each cell division requires that all DNA and the centrosome is replicated once per cell cycle and properly segregated into the newborn daughter cells. This is part of a strictly coordinated process of successive events which is referred to as the cell cycle. The duplication of the genetic information and its segregation into two daughter cells may be regarded as central processes of the cell cycle. Both events are separated from one another in higher eukaryotic cells (Howard and Pelc, 1951). The duplication of the chromosomes occurs in the synthesis or S phase, the separation and segregation of the two sister chromatids into two daughter nuclei occurs in mitosis. Both phases, the mitosis and DNA replication, are separated from one another by so-called gap phases: The phase before the synthesis phase is referred to as the G1 phase and the phase before mitosis is referred to as the G2 phase.

Cyclin-dependent kinases (CDKs) are the master regulators of the cell cycle control system. The amount and activity of these kinases ensures an orderly and uninterrupted progression through the cell cycle (Ekholm and Reed, 2000; Morgan, 1997; Pines and Rieder, 2001; Planas-Silva and Weinberg, 1997; Sherr, 1996). Specific kinases are activated and inactivated in a phase-specific manner during the course of the cell cycle. Oscillating CDK activity is a prerequisite for progression

through the cell cycle. Endogenous and internal checkpoints may regulate CDK kinase activity upon negative signals, for example due to lack of growth factors and these signals become thereby integrated into the cell cycle control system. The monomeric CDK subunits are catalytically inactive and have to associate with a positive regulatory subunit of the cyclin protein family in order to become activated. The abundance of most cell cycle regulatory cyclins is subjected to major variations during progression through the cell cycle. These oscillations of the cyclins plays one major role in the stage-specific activation and deactivation of CDKs. CDK kinase activity is further regulated by inhibitory and activating phosphorylation events and through CDK inhibitory proteins (CKIs).

CKIs of mammals are divided into two families according to their structure and their mechanism of action i.e. the Cip/Kip and the INK4 family (Carnero and Hannon, 1998; Hengst and Reed, 1998). The members of the Cip/Kip family, $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$ bind and inhibit a broad spectrum of cyclin/CDK complexes. They share a conserved amino-terminal domain which is necessary and sufficient for the inhibition of most cyclin/CDK complexes. Interestingly despite inhibiting CDKs, Cip/Kip proteins also can act as activators of cyclin D/CDK4,6 kinase complexes (LaBaer *et al.*, 1997; Cheng *et al.*, 1999 EMBO J 18, 1571). It is believed that they stimulate assembly of these complexes by binding to both subunits. Therefore, it has been reported that p21, p27 and p57 can associate with cyclin D/CDK4 complexes without inactivating them, however a number of studies have also reported inactivation of CDK4 kinase by p21 and p27.

The three-dimensional structure of the ternary complex of the inhibitor domain of p27 with CDK2 and the carboxy-terminal half of cyclin A have been elucidated by X-ray crystallography (Russo *et al.*, 1996a) and allows inferences to be made about the mechanism of inhibition: The amino-terminal region of the inhibitor domain of p27 binds the conserved cyclin box of cyclin A without significantly impairing its rigid structure. The carboxy-terminal region of the inhibitor domain interacts with

the amino-terminal domain of CDK2 and disturbs the conformation of its active site. In addition the inhibitor protrudes into the active centre of the kinase and thus blocks its ATP binding site. Even though the X-ray structure of p27 suggests that binding of p27 always leads to inactivation of the kinase complex, there are paradoxical observations that suggest that p27 or the related inhibitor p21 are required for activation of cyclin D/CDK4 complexes (LaBaer *et al.*, 1997; Cheng *et al.*, 1999 EMBO J 18, 1571).

The carboxy-terminal domains of the Cip/Kip proteins are different in size and only have slight sequence homologies between one another. The activity of the Cip/Kip proteins can be specifically regulated by modifications and protein-protein interactions of these carboxy-terminal domains (Hengst and Reed, 1998). For example phosphorylation of threonine 187 at the carboxy-terminal end has a major effect on the stability of p27 (Montagnoli *et al.*, 1999; Sheaff *et al.*, 1997). In addition p27 has a nuclear localization signal (NLS) in this region (Reynisdottir and Massague, 1997; Zeng *et al.*, 2000). A comparable NLS in the C-terminal domains of p21 and p57 is also responsible for their nuclear location.

The physiological and pathophysiological functions of p21 and p27 were and are the subject of intensive research. Both proteins have been characterized as ubiquitous negative regulators of cell proliferation since a number of endogenous and exogenous antiproliferative signals results in their expression in many different cell types. p21 is often involved in checkpoint controls, stress response and in the induction of differentiation processes whereas p27 seems to play a central role the control of the restriction point transition.

$p27^{Kip1}$ was discovered almost at the same time by several groups as a CDK-inhibiting activity in G1-arrested cell cultures (Hengst *et al.*, 1994; Polyak *et al.*, 1994a,b; Slingerland et al., 1994). Furthermore p27 was identified by a genetic screen as a protein binding to cyclin D1 (Toyoshima and Hunter, 1994). p27 is expressed

periodically in proliferating cells. level are at a maximum during the G1 phase, decrease strongly as soon as the cells enter the S phase and remains at a low level until the cells reach the next G1 phase (Hengst *et al.*, 1994; Hengst and Reed, 1996; Millard *et al.*, 1997). Moreover p27 is induced by a broad range of antiproliferative signals in many different cell types (Hengst and Reed, 1998). Thus, for example, p27 accumulates in cells which exit the cell cycle and become quiescent after withdrawing growth factors, or as a result of contact inhibition or removal of the substrate anchoring.

An abundance of experimental data indicates that the amount of p27 plays an important role in the regulation of the restriction point. For example most of the cyclin E/CDK2 and cyclin A/CDK2 complexes are inactive and if present associated with p27 in quiescent mouse fibroblasts. Hence inhibition of these complexes is due to p27 (Coats *et al.*, 1996). On the other hand the overexpression of p27 in cells results in an arrest in the G1 phase (Polyak et al., 1994b; Toyoshima and Hunter, 1994). Moreover reducing the amount of p27 using antisense RNA technology prevents fibroblasts from becoming quiescent after serum withdrawal. These fibroblasts also have a shortened G1 phase (Coats *et al.*, 1996; Rivard *et al.*, 1996). This phenotype is otherwise observed when G1 cyclins are overexpressed and is therefore consistent with the CDK inhibitor function of p27 (Ohtsubo *et al.*, 1995; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994; Resnitzky *et al.*, 1995; Resnitzky and Reed, 1995).

The role of p27 in cell cycle control has been confirmed by analysing p27-knockout mice. In two studies the p27 gene was completely deleted and in a third one it was replaced by a truncated p27 mutant lacking the CDK inhibitor domain (Fero *et al.*, 1996; Kiyokawa *et al.*, 1996; Nakayama *et al.*, 1996). All three mice strains have the same phenotype. It is mainly characterized by a gene dose-dependent increase in body size, general infertility of the female mice and deafness. The latter is caused by the continued proliferation of the hair cells of the corti organ in adult mice (Chen and

Segil, 1999; Lowenheim *et al.*, 1999). Apparently the absence of p27 interferes with the ability of a number of cell types to pass out of the cell cycle into the G0 phase or to differentiate during individual development (Vidal and Koff, 2000).

It is probable that p27 as a negative regulator of CDK activity plays a role as a tumour suppressor during the G1 phase. However, homozygotic inactivating mutations of the p27 gene are rarely found in human tumours (Kawamata *et al.*, 1995; Morosetti *et al.*, 1995; Pietenpol *et al.*, 1995; Spirin *et al.*, 1996). Therefore p27 is not a tumour suppressor in the classical sense (Knudson, 1971). However, remarkably low amounts of p27 are often detected in human tumours and these low amounts of p27 correlate with high tumour aggressiveness and high patient mortality (Slingerland and Pagano, 2000). A general increase in the incidence of tumours is not observed in p27-negative mice; however, the animals suffer from a change in the pituitary which has been classified as a benign adenoma (Nakayama *et al.*, 1996). Moreover p27-negative as well as p27-heterozygotic mice have an increased rate of tumours when irradiated or when treated with chemical carcinogens compared to the control group. This indicates a pivotal role of the amount of p27 in preventing tumours. p27 has been referred to as a "haplo-insufficient tumour suppressor", since no LOH has been found in tumours (Fero *et al.*, 1998).

The activity and amount of the inhibitor is of major importance for the function of p27. In this connection the expression of p27 can be regulated at various levels. In many cases it is not transcriptional but by means of the regulation of translation and stability of the proteins (Hengst and Reed, 1998).

p27-mRNA translation increases when normal diploid fibroblasts (HS68) leave the cell cycle as a result of contact inhibition. During the cell cycle, translation of p27-mRNA is subject to periodic oscillations. It is increased several times in HeLa cells arrested by lovastatin in the G1 phase compared to cells in the S phase (Hengst and Reed, 1996). A second mechanism of modulating the amount of a protein is to

regulate its proteolysis. p27 is degraded through the ubiquitin-proteasome pathway (Pagano et al., 1995). The ubiquitination of p27 occurs at the G1/S phase transition by the SCF-Skp2 ubiquitin ligase complex and CKS1 as a cofactor (Carrano *et al.*, 1999; Morimoto *et al.*, 2000; O'Hagan *et al.*; 2000; Spruck *et al.*, 2001; Sutterluty *et al.*, 1999; Tsvetkov *et al.*, 1999). p27 is more efficiently ubiquitinated and degraded in extracts of proliferating cells and cells in the S phase than in extracts of quiescent cells and cells in the G1 phase (Brandeis and Hunt, 1996; Montagnoli *et al.*, 1999; Nguyen *et al.*, 1999; Pagano *et al.*, 1995); the half live of the protein changes from 2,5 hours in G1 phase to less then 20 minutes in S phase (Hengst and Reed, 1996).

In late G1, S and G2 phase p27 must be phosphorylated at the threonine residue 187 by CDK2 in order to become a substrate of the SCF-SKp2 complex and to be subsequently degraded by the proteasome during the (Malek *et al.*, 2001; Montagnoli *et al.*, 1999; Muller *et al.*, 1997; Nguyen *et al.*, 1999; Sheaff *et al.*, 1997; Vlach *et al.*, 1997). During the G1 phase p27 is degraded by a second degradation pathway which is also ubiquitin-dependent but independent of the phosphorylation of threonine 187 (Malek *et al.*, 2001, Hara *et al.*, 2001). In this case the ubiquitination of p27 appears to take place in the cytoplasm.

Consistent with its central role in growth control, p27 was established as a marker for progression of various tumours. However, there are a number of exceptions where p27 fails as a tumour marker.

There is still a need to identify markers that can be used to analyse whether patients afflicted with cancer have a high risk for tumour progression. The tyrosine modification of p27 described here should allow one to determine whether p27 acts as a CDK inhibitor or activator, and thus be an excellent marker for tumour progression.

Summary of the invention

The embodiments of the present invention are based on the finding that the CDK inhibitor protein p27Kip1 becomes phosphorylated on tyrosine residues 88 and 89 by tyrosine directed protein kinases. This modification has three potential consequences:

- The inhibitory activity of p27 is reduced.

- The inhibitor becomes a better substrate for CDK2-dependent phosphorylation that triggers its SCF-Skp2-dependent degradation.

- The modified inhibitor acts as an activator of cyclin-dependent kinases by exerting CDK/cyclin assembly factor activity.

The eukaryotic cell cycle is regulated by the oscillating activity of various cyclin-dependent kinases (CDKs). CDK kinase activity is regulated by CDK inhibitor proteins. The amount of the CDK inhibitor protein $p27^{Kip1}$ plays a key role in the transition of the cell from the G1 to the S phase. The amount of $p27^{Kip1}$ increases during the G0 or the G1 phase and decreases again rapidly at the onset of the S phase. The binding of $p27^{Kip1}$ to the CDK2 kinase complexes in the G1 phase inactivates them and can thus prevents the initiation of the S phase. A reduced amount of $p27^{Kip1}$ at the G1/S phase transition is frequently found in various tumour tissues. In this connection the smaller amount of the inhibitor is associated with a high patient mortality and an aggressive course of the disease. The SH3 domain protein Grb2 was identified as interaction partner of the inhibitor $p27^{Kip1}$. This interaction involves the C-terminal SH3 domain of Grb2 and a proline-rich region in p27. In search for other SH3 domains that are able to associate with the proline-rich domain in p27, it was discovered the src-related tyrosine kinase Lyn as a binding partner. Using recombinant tyrosine kinases, it was discovered that p27 is a substrate for tyrosine kinases including scr, Abl, Bcr-Abl or Lyn. Some tyrosine kinases may

interact with p27 by using their SH3 domains or the adapter function of Grb2, however SH3- independent mechanisms may direct tyrosine kinases to p27. These may not involve a direct interaction between the inhibitor and the kinase. It was demonstrated initially by phosphoamino acid analysis and later using phospho-specific antibodies that p27 and p21 become phosphorylated on tyrosine *in vivo*. Mutational analysis and later generation of phospo-specific antibodies allowed to determine that preferably tyrosine residue 88 but also tyrosine 89 are modified by Bcr-Abl and Lyn *in vitro* and *in vivo*. Tyrosine residue 88 of p27 is a substrate for Abl, Bcr-Abl, Lyn and src kinases.

According to crystal structural data of the trimeric complex consisting of $p27^{Kip1}$, CDK2 and cyclin A, the tyrosine residue 88 of $p27^{Kip1}$ becomes situated in the ATP binding pocket of the kinase and blocks it. Hence it was examined to what extent a phosphorylation of $p27^{Kip1}$ on tyrosine 88 or 89 influences the activity of the inhibitor. The tyrosine phosphorylation of $p27^{Kip1}$ does not prevent binding to the CDK complex. However, using in vitro-phosphorylated $p27^{Kip1}$ it was shown that a tyrosine phosphorylation leads to a reduction of the activity of the inhibitor.

The negative charge of the phosphate group on tyrosine 88 may interfere with the positioning of the tyrosine residue in the purine binding pocket of the CDK. This allows the inhibitor to promote activation of the kinase by promoting CDK/cyclin D assembly, converting the inhibitor into an activator of cyclin-dependent kinases. This reduction may explain the contradictory observations of the inhibition of cyclinD / CDK4 inhibition by p21 or p27 proteins: After phosphorylation of the tyrosine 88 residue, the ability of p27 or p21 to inhibit CDKs is substantially reduced. By binding to both kinase subunits, CDK4,6 and D-type cyclins modified p27 may stimulate complex assembly of this kinase.

It is interesting that the tyrosine phosphorylation of the inhibitor significantly increases the phosphorylation of $p27^{Kip1}$ at threonine 187 by the bound CDK

complex. The phosphorylation of $p27^{Kip1}$ at threonine 187 is an initial signal to degrade $p27^{Kip1}$ by the 26S proteosome. Threonine 187-phosphorylated $p27^{Kip1}$ is recognized by the E3-ligase complex SCF-Skp2/Cks1 and ubiquitinated. Therefore tyrosine phosphorylation of p27 may initiate the ubiquitin-dependent degradation of the inhibitor through the SCF-Skp2 complex and the proteasome at the G1/S transition.

Consistent with its central role in growth control, it was found that p27 tyrosine phosphorylation is enhanced in cells transfected with Bcr-abl, src or Lyn tyrosine kinases. Increased tyrosine phosphorylated p27 should be a valuable marker in various tumours. The detection of tyrosine-phosphorylated p27 or the ratio of tyrosine-phosphorylated versus non-modified p27 could be used as a prognostic or monitoring marker in tumours. A mutant p27 was generated where tyrosines 88 and 89 have been substituted by phenylalanine. The mutant protein still acts as a CDK inhibitor. This mutant protein can be used as a modification-resistant CDK inhibitor that cannot be inactivated or converted into an activator by tyrosine kinases. If used in therapy, the CDK inhibitory domain of p27 will be significantly improved over the wild-type CDK inhibitory domain. Since the tyrosine residue 88 of p27 is highly conserved in the related inhibitors p21 and p57, these inhibitors can also be regulated by tyrosine phosphorylation.

Therefore, in an embodiment of the invention, a polypeptide is provided comprising the amino acid sequence SEQ ID NO: 2, 4 or 6 whereby in the polypeptide comprising the amino acid sequence SEQ ID NO: 2, the tyrosine residue at position 88 and/ or the tyrosine residue at position 89 in SEQ ID NO: 2 is/ are phosphorylated, or whereby in the polypeptide comprising the amino acid sequence SEQ ID NO: 4, the tyrosine residue at position 77 in SEQ ID NO 4 is phosphorylated, or whereby in the polypeptide comprising the amino acid sequence SEQ ID NO: 6, the tyrosine residue at position 91 in SEQ ID NO 6 is phosphorylated.

In another embodiment of the invention, a peptide fragment is provided with a minimum length of 6 amino acids of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, 4 or 6, whereby in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, the tyrosine residue at position 88 and/ or the tyrosine residue at position 89 in SEQ ID NO: 2 is/ are phosphorylated, or whereby in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 4, the tyrosine residue at position 77 in SEQ ID NO 4 is phosphorylated, or whereby in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 6, the tyrosine residue at position 91 in SEQ ID NO 6 is phosphorylated.

In another embodiment of the invention, a non-phosphorylable polypeptide is provided comprising the amino acid sequence SEQ ID NO: 2, 4 or 6 or
comprising the amino acid residues 1 to 95 of SEQ ID NO: 2 or comprising the amino acid residues 1 to 85 of SEQ ID NO: 4 or comprising the amino acid residues 1 to 100 of SEQ ID NO: 6 or
comprising the amino acid residues 50 to 95 of SEQ ID NO: 2 or comprising the amino acid residues 38 to 85 of SEQ ID NO: 4 or comprising the amino acid residues 50 to 100 of SEQ ID NO: 6
characterized in that
in the polypeptide derived from the polypeptide with the amino acid sequence SEQ ID NO: 2, the amino acid residue at position 88 and/or position 89 in SEQ ID NO: 2, or
in the polypeptide derived from the polypeptide with the amino acid sequence SEQ ID NO: 4, the amino acid residue at position 77 in SEQ ID NO: 4 or
in the polypeptide derived from the polypeptide with the amino acid sequence SEQ ID NO: 6, the amino acid residue at position 91 in SEQ ID NO: 6
is a non-phosphorylable amino acid residue, preferably a phenylalanine residue.

In another embodiment of the invention, a non-phosphorylable peptide fragment is provided with a minimum length of 6 amino acids of a polypeptide comprising the

amino acid sequence SEQ ID NO: 2, 4 or 6
characterized in that
in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, the peptide fragment comprises at least one of the flanking amino acid residues of the amino acid residue at position 88 or 89 in SEQ ID NO: 2 and the residue at position 88 and/ or the residue at position 89 in SEQ ID NO: 2 is a non-phosphorylable amino acid residue, preferably a phenylalanine residue, or
in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 4, the peptide fragment comprises at least one of the flanking amino acid residues of the amino acid residue at position 77 in SEQ ID NO: 4 and the residue at position 77 in SEQ ID NO: 4 is a non-phosphorylable amino acid residue, preferably a phenylalanine residue, or
in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 6, the peptide fragment comprises at least one of the flanking amino acid residues of the amino acid residue at position 91 in SEQ ID NO: 6 and the residue at position 91 in SEQ ID NO: 6 is a non-phosphorylable amino acid residue, preferably a phenylalanine residue.

In yet another embodiment of the invention, a nucleic acid molecule is provided encoding a polypeptide according to the invention or a peptide fragment according to the invention.

In still another embodiment, an isolated antibody is provided which specifically binds to a polypeptide according to the invention or to a peptide fragment according to the invention and which has less than 10% cross reactivity with the non-phosphorylated polypeptide or non-phosphorylated peptide fragment.

In an embodiment of the invention, a polypeptide according to the invention, a peptide fragment according to the invention, a nucleic acid molecule according to the invention or an antibody according to the invention is provided for use in medicine or may be contained in a pharmaceutical composition.The said substances according

to the invention may also be used for the preparation of a pharmaceutical composition for the treatment of hyperproliferative disease, preferably cancer.

In still another embodiment of the invention, a method is provided for the determination of the amount or presence of a polypeptide according to the invention or a peptide fragment according to the invention in a sample comprising the steps of
a) providing a sample suspected to contain the polypeptide or the peptide fragment,
b) incubating the sample in the presence of an antibody according to the invention, and
c) determining the binding product between the polypeptide and the antibody thereby concluding that the polypeptide or peptide fragment is present or thereby deriving the amount of the polypeptide or the peptide fragment.

In comparison to the normal level of the polypeptide or peptide fragment according to the invention and further variations, this method may also be used for determining whether or not a human cancer cell containing patient sample has potential for tumor progression, selecting a composition for inhibiting the progression of cancer in a patient and deriving a candidate agent. Further, the polypeptide according to the invention or a peptide fragment according to the invention may be used as an immunogen to generate or produce antibodies, in particular monoclonal antibodies.

In yet another embodiment of the invention, a kit for the detection or determination of the amount of the polypeptide according to the invention or the peptide fragment according to the invention in a biological sample is provided which comprises:
(a) an antibody according to the invention and
(b) a label for qualitatively or quantitatively detecting an immunoconjugate of the antibody and the polypeptide or the peptide fragment.

Detailed description of the invention

The one letter code for amino acids is used often throughout this application which can be found in standard textbooks. The most important abbreviations for this application is Y for TYR or tyrosine and F for PHE or phenylalanine. The abbreviations are A, Ala: Alanine, R, Arg: Arginine, N,Asn: Asparagine, D,Asp: Aspartic acid, C,Cys: Cysteine, Q, Gln: Glutamine, E, Glu: Glutamic acid, G, Gly: Glycine, H, His: Histidine, I, Ile: Isoleucine, L, Leu: Leucine, K, Lys: Lysine, M, Met: Methionine, F, Phe: Phenylalanine, P, Pro: Proline, S, Ser: Serine, T, Thr: Threonine, W, Trp: Tryptophan, Y, Tyr: Tyrosine, V, Val: Valine.

I. Isolated Proteins and Antibodies

The invention provides a recombinant polypeptide or protein with an amino acid sequence according to SEQ ID NO: 2, 4 or 6 and which can be encoded by the DNA sequence shown in SEQ ID NO: 1, 3 or 5 and which is phosphorylated at the tyrosine residues 88 and/or 89 or at the positions in p21 and p57 that correspond to tyrosine residue 88 in p27 that can be identified by sequence alignment (see Fig. 1). The sequence alignment in Fig. 1 also allows identifying other corresponding amino acids or functional elements of the polypeptides.

In an embodiment of the invention, phosphorylated forms of p21, p27 and p57 are contemplated, i.e. a polypeptide is provided comprising the amino acid sequence SEQ ID NO: 2, 4 or 6 whereby in the polypeptide comprising the amino acid sequence SEQ ID NO: 2, the tyrosine residue at position 88 and/ or the tyrosine residue at position 89 in SEQ ID NO: 2 is/ are phosphorylated, or
whereby in the polypeptide comprising the amino acid sequence SEQ ID NO: 4, the tyrosine residue at position 77 in SEQ ID NO 4 is phosphorylated, or
whereby in the polypeptide comprising the amino acid sequence SEQ ID NO: 6, the tyrosine residue at position 91 in SEQ ID NO 6 is phosphorylated. An embodiment

of the invention specifically contemplates fusion proteins with other partners. Preferably, the polypeptide consists of the amino acid sequence SEQ ID NO: 2, 4 or 6 whereby the said tyrosines are phosphorylated. The term "are/ is phosphorylated" means in other words in the context of the invention that if the tyrosine is phosphorylated, it is a phosphotyrosine residue or a phosphodiester between H_3PO_4 and the (substituted) phenol alcohol moiety of the tyrosine.

In another embodiment of the invention, even further phosphorylated forms of p27 are provided, i.e. a polypeptide comprising the amino acid sequence SEQ ID NO: 2, whereby the tyrosine residues at position 88 and/ or the tyrosine residue at position 89 in SEQ ID NO: 2 are phosphorylated and whereby the serine residue at position 10 and/or 12 and/or the threonine residue at position 157 and/or the threonine residue at position 187 in SEQ ID NO: 2 are phosphorylated. It is preferred that only position 187 is phosphorylated. An embodiment of the invention specifically contemplates fusion proteins with other partners. Preferably, the polypeptide consists of the amino acid sequence SEQ ID NO: 2 whereby at least one of the said tyrosines is phosphorylated.

In other embodiments of the invention, a peptide fragment is provided with a minimum length of 6 amino acids of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, 4 or 6, whereby in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, the tyrosine residue at position 88 and/ or the tyrosine residue at position 89 in SEQ ID NO: 2 is/ are phosphorylated, or whereby in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 4, the tyrosine residue at position 77 in SEQ ID NO 4 is phosphorylated, or whereby in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 6, the tyrosine residue at position 91 in SEQ ID NO 6 is phosphorylated. The length of 6 amino acids corresponds to an epitope. In a more preferred embodiment of the invention, the peptide fragment is provided with a minimum length of 8, more preferably 10 or 12 amino acids in particular where the phosphorylated tyrosines are located in the middle of the peptide

fragment. Preferably, the peptide fragment has a minimum length of 15 amino acids. Overall the peptide fragment may be up to 30 amino acids long. An embodiment of the invention specifically contemplates fusion peptides with other partners. Preferably, the peptide fragments only consists of fragments of the amino acid sequences SEQ ID NO: 2, 4 or 6 whereby at least one of the said tyrosines is phosphorylated.

Preferred peptide fragments of p27 have the amino acid sequence EKGSLPEFYYRPPRP (15 amino acids (AA), SLPEFYYRPPRP (12 amino acids (AA)), LPEFYYRPPR (10 amino acids (AA)), PEFYYRPP (8 amino acids (AA)) or EFYYRP (6 amino acids (AA)) whereby at least one of the tyrosines is phosphorylated. Preferred p21 and p57 peptide fragments can be identified accordingly by identifying the corresponding amino acid residues from the sequence alignment in Fig. 1.

The peptide fragments are particularly useful to be used as an antigen for producing antibodies, particularly monoclonal antibodies.

The invention also discloses polypeptides, fragments thereof as the CDK inhibitory or binding domains or peptide fragments where the said tyrosine is preferably substituted by phenylalanine or any other amino acid to make the protein or polypeptide resistant to tyrosine modification at the described positions, i.e. phosphorylation, whereby in p27 only one tyrosine may be exchanged making it resistant to phosphorylation by protein kinases only phosphorylating at the indicated position. Therefore, the amino acid residue tyrosine should be absent, preferably also the amino acids serine, threonine or the amino acids with carboxylate carrying side chains as aspartate and glutamate which mimick a phosphate group. The preferred exchange is phenylalanine but is evident for an expert skilled in the art that other amino acid residues may be incorporated as well.

Therefore, in a preferred embodiment of the invention, a non-phosphorylable polypeptide is provided comprising the amino acid sequence SEQ ID NO: 2, 4 or 6

or comprising the amino acid residues 1 to 95 of SEQ ID NO: 2 or comprising the amino acid residues 1 to 85 of SEQ ID NO: 4 or comprising the amino acid residues 1 to 100 of SEQ ID NO: 6 or comprising the amino acid residues 50 to 95 of SEQ ID NO: 2 or comprising the amino acid residues 38 to 85 of SEQ ID NO: 4 or comprising the amino acid residues 50 to 100 of SEQ ID NO: 6 characterized in that in the polypeptide derived from the polypeptide with the amino acid sequence SEQ ID NO: 2, the amino acid residue at position 88 and/or position 89 in SEQ ID NO: 2, or in the polypeptide derived from the polypeptide with the amino acid sequence SEQ ID NO: 4, the amino acid residue at position 77 in SEQ ID NO: 4 or in the polypeptide derived from the polypeptide with the amino acid sequence SEQ ID NO: 6, the amino acid residue at position 91 in SEQ ID NO: 6 is a non-phosphorylable amino acid residue, preferably a phenylalanine residue. An embodiment of the invention specifically contemplates fusion proteins with other partners. Preferably, the polypeptide consists of the amino acid sequence SEQ ID NO: 2, 4 or 6 whereby at least one of the said tyrosines is phosphorylated.

In another preferred embodiment of the invention, a peptide fragment is provided with a minimum length of 6 amino acids of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, 4 or 6
characterized in that
in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, the peptide fragment comprises at least one of the flanking amino acid residues of the amino acid residue at position 88 or 89 in SEQ ID NO: 2 and the residue at position 88 and/ or the residue at position 89 in SEQ ID NO: 2 is a non-phosphorylable amino acid residue, preferably a phenylalanine residue, or
in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 4, the peptide fragment comprises at least one of the flanking amino acid residues of the amino acid residue at position 77 in SEQ ID NO: 4 and the residue at position 77 in SEQ ID NO: 4 is a non-phosphorylable amino acid residue, preferably a phenylalanine residue, or

in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 6, the peptide fragment comprises at least one of the flanking amino acid residues of the amino acid residue at position 91 in SEQ ID NO: 6 and the residue at position 91 in SEQ ID NO: 6 is a non-phosphorylable amino acid residue, preferably a phenylalanine residue. In a more preferred embodiment of the invention, the peptide fragment is provided with a minimum length of 8, more preferably 10 or 12 amino acids in particular where the residues exchanged for the phosphorylatable tyrosines are located in the middle of the peptide fragment. Preferably, the peptide fragment has a minimum length of 15 amino acids. Overall the peptide fragment may be up to 30 amino acids long. An embodiment of the invention specifically contemplates fusion peptides with other partners. Preferably, the peptide fragments only consists of fragments of the amino acid sequences SEQ ID NO: 2, 4 or 6 whereby at least one of the said tyrosines are exchanged for a non-phosphorylable amino acids, preferably phenylalanin.

Preferred non-phosphorylable peptide fragments of p27 have the amino acid sequence EKGSLPEF(Y/F)(Y/F)RPPRP (15 amino acids (AA), SLPEF(Y/F)(Y/F)RPPRP (12 amino acids (AA)), LPEF(Y/F)(Y/F)RPPR (10 amino acids (AA)), PEF(Y/F)(Y/F)RPP (8 amino acids (AA)) or EF(Y/F)(Y/F)RP (6 amino acids (AA)) whereby at least one of the tyrosines is substituted by phenylalanine as denoted by (Y/F). Preferred p21 and p57 peptide fragments can be identified accordingly by identifying the corresponding amino acid residues from the sequence alignment in Fig. 1.

The invention further contemplates a method for producing a polypeptide or peptide fragment according to the invention by expressing an exogenous DNA in prokaryotic or eukaryotic host cells and isolation of the desired protein, wherein the protein is preferably coded by the nucleic acid sequence as shown in SEQ ID NO:1. The protein can be isolated from the cells or the culture supernatant and purified by chromatographic means, preferably by ion exchange chromatography, affinity chromatography and/or reverse phase HPLC. The isolated protein is then

phosphorylated using the described, isolated and commercially available protein kinases including the src family kinases, Abl kinases, receptor-associated kinases. In more detail in order to generate p27 protein phosphorylated on tyrosyl side chains the p27 cDNA is expressed in E.coli by cloning the cDNA into an eucaryotic expression vector, e.g. pET8c. Expression of p27 is induced with IPTG at 37°C for up to 4 hours. Recombinant p27 protein is purified by heat treatment, anion and cation exchange chromatography and gel filtration as described in methods 3.4.8 to 3.4.11. Tyrosine kinases like Abl, Src and Lyn are expressed and purified from E.coli and purified as described in 3.4.8 to 3.4.11 or sold from commercial suppliers. p27 was phosphorylated in vitro by mixing recombinant p27 and the appropriate tyrosine kinase in a buffer containing 25uM ATP, 200mM Tris-HCl pH 7.2 and 75mM MgCl2 as described in 3.4.13.

The isolated polypeptide or peptide fragment according to the invention can occur in natural allelic variations which differ from individual to individual. Such variations of the amino acids are usually amino acid substitutions. However, they may also be deletions, insertions or additions of amino acids to the total sequence. The polypeptide or peptide fragment according to the invention - depending, both in respect of the extent and type, on the cell and cell type in which it is expressed- can be in glycosylated or non-glycosylated form. This will be described in more detail below.

"Polypeptide with p27 activity" means also proteins with minor amino acid variations but with substantially the same activity. Substantially the same means that the activities are of the same biological properties and the polypeptides show (at least 90 %, preferably more than 95 %) homology or preferably identity in amino acid sequence. Homology can be examined by using the BLAST algorithm described by Altschul, S.F., *et al.*, Nucleic Acids Res. 25 (1997) 3389-3402. This is also described in more detail below.

The polypeptide or peptide fragment according to the invention can be produced by recombinant means in host cells using an expression vector or can be produced synthetically. Non-glycosylated p27 polypeptide is obtained when it is produced recombinantly in prokaryotes. The p27 polypeptide or peptide fragment can be purified after recombinant production by affinity chromatography using known protein purification techniques including immunoprecipitation, gel filtration, ion exchange chromatography, chromato-focussing, isoelectric focussing, selective precipitation, electrophoresis or the like. This will be described in more detail below.

One aspect of the invention concerns isolated phosphorylated or phosphorylable proteins encoded by the nucleic acid sequence of the invention and biologically active portions thereof, as well as polypeptide fragments suitable for use as immunogens to raise antibodies directed against a polypeptide encoded by the nucleic acid sequence. In one embodiment the native polypeptide encoded by the nucleic acid sequence can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques. In another embodiment a polypeptide or peptide fragment encoded by the nucleic acid sequence of the invention is produced by recombinant DNA techniques. Alternative to recombinant expression, a polypeptide or peptide fragment encoded by the nucleic acid sequence according to the invention can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" polypeptide or peptide fragment or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived or substantially free of chemical precursors or other chemicals when chemically synthesized. The term "substantially free of cellular material" includes preparations of polypeptide or peptide fragment in which the polypeptide or peptide fragment is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus a protein that is substantially free of cellular material

includes preparations of protein having less than about 30 %, 20 %, 10 %, or 5 % (by dry weight) of heterologous protein (also referred to herein as a "contaminating protein").

When the polypeptide or peptide fragment or biologically active portion thereof is recombinantly produced it is also preferably substantially free of culture medium i. e. culture medium represents less than about 20 %,10 % or 5 % of the volume of the protein preparation. When the protein is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals i. e. it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. Accordingly such preparations of the protein have less than about 30 %, 20 %, 10 %, 5 % (by dry weight) of chemical precursors or compounds other than the polypeptide of interest.

Biologically active portions of a polypeptide or peptide fragment encoded by a nucleic acid molecule according to the invention include polypeptide or peptide fragment comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the protein according to the invention which include fewer amino acids than the full length protein and exhibit at least one activity of the corresponding full-length protein. Typically biologically active portions comprise a domain or motif with at least one activity of the corresponding protein. A biologically active portion of the polypeptide or peptide fragment of the invention can be a polypeptide which is for example 10, 25, 50, 100 or more amino acids in length depending on the total length of the polypeptide or peptide fragment.

Moreover, other biologically active portions in which other regions of the polypeptide or peptide fragment are deleted can be prepared by recombinant techniques and evaluated for one or more of the functional activities of the native form of a polypeptide of the invention. Other useful proteins are substantially identical (e.g. at least about 40 %, preferably 50 %, 60 %, 70 %, 80 %, 90 %, 95 %,

or 99 %) to one of these sequences and retain the functional activity of the polypeptide or peptide fragment of the corresponding naturally-occurring polypeptide or peptide fragment yet differ in amino acid sequence due to natural allelic variation or mutagenesis.

To determine the percent identity of two amino acid sequences or of two nucleic acids the sequences are aligned for optimal comparison purposes (e.g. gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e. % identity = of identical positions/total of positions (e.g. overlapping positions) x 100). In one embodiment the two sequences are the same length. The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin, S., and Altschul, S.F., Proc. Natl. Acad. Sci. USA 87 (1990) 2264-2268, modified as in Karlin, S., and Altschul, S.F., Proc. Natl. Acad. Sci. USA 90 (1993) 5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, S.F., *et al.*, J. Mol. Biol. 215 (1990) 403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, word length = 12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, word length = 3 to obtain amino acid sequences homologous to a protein molecule of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul, S.F., et al., Nucleic Acids Res. 25 (1997) 3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships

between molecules. When utilizing BLAST, Gapped BLAST and PSI-Blast programs the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov. Another preferred non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, (1988) CABIOS or 11 - 17. Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 can be used. Yet another useful algorithm for identifying regions of local sequence similarity and alignment is the FASTA algorithm as described in Pearson, W.R., and Lipman, D.J., Proc. Natl. Acad. Sci. USA 85 (1988) 2444-2448. When using the PASTA algorithm for comparing nucleotide or amino acid sequences, a PAM120 weight residue table can for example be used with a k-tuple value of 2. The percent identity between two sequences can be determined using techniques similar to those described above with or without allowing gaps. In calculating percent identity only exact matches are counted.

The invention also provides chimeric or fusion polypeptides or proteins corresponding to the polypeptide or peptide fragment according to the invention. As used herein, a "chimeric protein" or "fusion protein" comprises all or part (preferably a biologically active part) of the polypeptide or peptide fragment according to the invention operably linked to a heterologous polypeptide (i e. a polypeptide other than the polypeptide encoded by the nucleic acid molecule). Within the fusion protein the term "operably linked" is intended to indicate that the polypeptide of the invention and the heterologous polypeptide are fused in-frame to each other. The heterologous polypeptide can be fused to the amino-terminus or the carboxyl-terminus of the polypeptide of the invention. One useful fusion protein is a GST fusion protein in which a polypeptide according to the invention is fused to the carboxyl terminus of GST sequences. Such fusion proteins can facilitate the purification of a recombinant polypeptide or peptide fragment of the invention. In another embodiment the fusion

protein contains a heterologous signal sequence at its amino terminus. For example the native signal sequence of a polypeptide according to the invention can be removed and replaced with a signal sequence from another protein. For example the gp67 secretory sequence of the baculovirus envelope protein can be used as a heterologous signal sequence (Ausubel *et al.*, ed., Current Protocols in Molecular Biology, John Wiley & Sons, NY, 1992). Other examples of eukaryotic heterologous signal sequences include the secretory sequences of melittin and human placental alkaline phosphatase (Stratagene; La Jolla, California). In yet another example useful prokaryotic heterologous signal sequences include the phoA secretory signal (Sambrook *et al.*, supra) and the protein A secretory signal (Pharmacia Biotech; Piscataway, New Jersey). Cell permeable fusion proteins to deliver mutant p27 or CKI domains into cells may also be generated. These fusions may include a peptide from the TAT protein of HIV, a domain of the antennapedia protein (Antp) from drosophila or HSV VP22 protein from HSV. An example of cell permeable p27 fusion proteins is described in Nagahara et al., 1998 (Nature Medicine Vol4 Nr.12 page 1449-1452). The TAT peptide used here is the N-terminal tag YGRKKRRQRRR.

In yet another embodiment the fusion protein is an immunoglobulin fusion protein in which all or part of a polypeptide according to the invention is fused to sequences derived from a member of the immunoglobulin protein family. The immunoglobulin fusion proteins of the invention can be used as immunogens to produce antibodies directed against a polypeptide of the invention in a subject, to purify ligands and in screening assays to identify molecules which inhibit the interaction of receptors with ligands.

Chimeric and fusion proteins of the invention can be produced by standard recombinant DNA techniques. In another embodiment the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers.

Alternatively the PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed and re-amplified to generate a chimeric gene sequence (see e.g. Ausubel *et al.*, supra). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g. a GST polypeptide).

A nucleic acid encoding a polypeptide of the invention can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the polypeptide or peptide fragment of the invention.

A signal sequence can be used to facilitate secretion and isolation of the secreted protein or other proteins of interest. Signal sequences are typically characterized by a core of hydrophobic amino acids which are generally cleaved from the mature protein during secretion in one or more cleavage events. Such signal peptides contain processing sites that allow cleavage of the signal sequence from the mature proteins as they pass through the secretory pathway. Thus the invention pertains to the described polypeptides having a signal sequence, as well as to polypeptides from which the signal sequence has been proteolytically cleaved (i. e. the cleavage products). In one embodiment a nucleic acid sequence encoding a signal sequence can be operably linked in an expression vector to a protein of interest, such as a protein which is ordinarily not secreted or is otherwise difficult to isolate. The signal sequence directs secretion of the protein, such as from a eukaryotic host into which the expression vector is transformed and the signal sequence is subsequently or concurrently cleaved. The protein can then be readily purified from the extracellular medium by art recognized methods.

Alternatively the signal sequence can be linked to the protein of interest using a sequence which facilitates purification such as with a GST domain.

The present invention also pertains to variants of the polypeptide or peptide fragment encoded by nucleic acid molecule of the invention. Such variants have an altered amino acid sequence which can function as either agonists (mimetics) or as antagonists. Variants can be generated by mutagenesis e.g. discrete point mutation or truncation. An agonist can retain substantially the same, or a subset of the biological activities of the naturally occurring protein. An antagonist of a protein can inhibit one or more of the activities of the naturally occurring form of the protein by for example competitively binding to a downstream or upstream member of a cellular signalling cascade which includes the protein of interest. Thus specific biological effects can be elicited by treatment with a variant of limited function. Treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the protein can have fewer side effects in a subject relative to treatment with the naturally occurring form of the protein.

Further variants of a polypeptide or peptide fragment of the invention which function as either agonists (mimetics) or as antagonists can be identified by screening combinatorial libraries of mutants e.g. truncation mutants of the protein of the invention for agonist or antagonist activity. In one embodiment a variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of variants can be produced by for example enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential protein sequences is expressible as individual polypeptides or alternatively as a set of larger fusion proteins (e.g. for phage display). There are a variety of methods which can be used to produce libraries of potential variants of the polypeptides of the invention from a degenerate oligonucleotide sequence. Methods for synthesizing degenerate oligonucleotides are known in the art (see e.g. Narang, Tetrahedron 39 (1983) 3; Itakura, K., *et al.*, Annu. Rev. Biochem. 53 (1984) 323-356; Itakura *et al.*, 1984, Science 198:1056; Ike, Y., *et al.*, Nucleic Acid Res. 11 (1983) 477-488).

In addition libraries of fragments of the coding sequence of a polypeptide encoded by the nucleic acid molecule according to the invention can be used to generate a variegated population of polypeptides for screening and subsequent selection of variants. A library of coding sequence fragments can for example be generated by treating a double-stranded PCR fragment of the coding sequence of interest with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double-stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease and ligating the resulting fragment library into an expression vector. By this method an expression library can be derived which encodes amino terminal and internal fragments of various sizes of the protein of interest.

Several techniques are known in the prior art for screening gene products of combinatorial libraries made by point mutations or truncation and for screening cDNA libraries for gene products having a selected property. The most widely used techniques which are amenable to high through-put analysis for screening large gene libraries typically include cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can be used in combination with the screening assays to identify variants of a protein of the invention (Arkin, A.P., and Youvan, D.C., Proc. Natl. Acad. Sci. USA 89 (1992) 7811-7815; Delagrave, S., *et al.*, Prot. Eng. 6 (1993) 327-331).

An isolated polypeptide according to the invention or a fragment thereof can be used as an immunogen to generate antibodies using standard techniques for polyclonal and monoclonal antibody preparation. The full-length polypeptide or protein can be used

or alternatively the invention provides antigenic peptide fragments for use as immunogens. The antigenic peptide fragment of a polypeptide of the invention comprises at least 6, 8 (preferably 10, 15, 20, or 30 or more) amino acid residues of the amino acid sequence of the polypeptide of the invention and encompasses an epitope of the protein comprising the phosphorylated amino acid residues Y 88 and/or Y 89 such that an antibody raised against the peptide forms a specific immune complex with a protein according to the invention. Preferred epitopes encompassed by the antigenic peptide are regions that are located on the surface of the protein e.g. hydrophilic regions. Hydrophobicity sequence analysis, hydrophilicity sequence analysis or similar analyses can be used to identify hydrophilic regions. An immunogen typically is used to prepare antibodies by immunizing a suitable (i.e. immunocompetent) subject such as a rabbit, goat, mouse or other mammal or vertebrate. An appropriate immunogenic preparation can for example contain recombinantly-expressed or chemically-synthesized polypeptide. The preparation can further include an adjuvant, such as Freund's complete or incomplete adjuvant or a similar immunostimulatory agent.

Therefore, in an embodiment of the invention, a polypeptide according to the invention or a peptide fragment, in particular the phosphorylated proteins or peptide fragments, can be used as an immunogen to generate or produce antibodies using standard techniques for polyclonal and monoclonal antibody preparation. The standard techniques are described herein and are known to an expert skilled in the art.

Accordingly another aspect of the invention pertains to antibodies directed against a polypeptide of the invention. The terms "antibody" and "antibody substance" as used interchangeably herein refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules i. e. molecules that contain an antigen binding site which specifically binds an antigen, such as a polypeptide of the invention e.g. an epitope of a polypeptide of the invention. A molecule which

specifically binds to a given polypeptide of the invention is a molecule which binds the polypeptide, but does not substantially bind other molecules in a sample e.g. a biological sample which naturally contains the polypeptide. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. The invention provides polyclonal and monoclonal antibodies. The term "monoclonal antibody" or "monoclonal antibody composition" as used herein refers to a population of antibody molecules that contain only one species of an antigen binding site capable of immunoreacting with a particular epitope.

Polyclonal antibodies can be prepared as described above by immunizing a suitable subject with a polypeptide or peptide fragment of the invention as an immunogen as also shown in the example part of this application. Preferred polyclonal antibody compositions are ones that have been selected for antibodies directed against a polypeptide or polypeptides of the invention. Particularly preferred polyclonal antibody preparations are ones that contain only antibodies directed against a polypeptide or polypeptides of the invention. Particularly preferred immunogen compositions are those that contain no other human proteins such as for example immunogen compositions made using a non-human host cell for recombinant expression of a polypeptide of the invention. In such a manner the only human epitope or epitopes recognized by the resulting antibody compositions raised against this immunogen will be present as part of a polypeptide or polypeptides of the invention.

The antibody titre in the immunized subject can be monitored over time by standard techniques such as with an enzyme linked immunosorbent assay (ELISA) using immobilized polypeptide. If desired the antibody molecules can be harvested or isolated from the subject (e.g. from the blood or serum of the subject) and further purified by well-known techniques such as protein A chromatography to obtain the IgG fraction. Alternatively antibodies specific for a protein or polypeptide of the

invention can be selected or (e.g. partially purified) or purified by e.g. affinity chromatography.

For example a recombinantly expressed and purified (or partially purified) polypeptide or peptide fragment of the invention is produced as described herein and covalently or non-covalently coupled to a solid support such as for example a chromatography column. The column can then be used to affinity purify antibodies specific for the proteins of the invention from a sample containing antibodies directed against a large number of different epitopes, thereby generating a substantially purified antibody composition i.e. one that is substantially free of contaminating antibodies. By a substantially purified antibody composition is meant in this context that the antibody sample contains at most only 30 % (by dry weight) of contaminating antibodies directed against epitopes other than those of the desired protein or polypeptide of the invention and preferably at most 20 % yet more preferably at most 10 % and most preferably at most 5 % (by dry weight) of the sample is contaminating antibodies. A purified antibody composition means that at least 99 % of the antibodies in the composition are directed against the desired protein or polypeptide of the invention.

At an appropriate time after immunization e.g. when the specific antibody titres are highest antibody-producing cells can be obtained from the subject and used to prepare monoclonal antibodies by standard techniques, such as the hybridoma technique originally described by Kohler, G., and Milstein, C., Nature 256 (1975) 495-497 the human B cell hybridoma technique (see Kozbor *et al.*, Immunol. Today 4 (1983) 72), the EBV hybridoma technique (see Cole *et al.*, In: Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., 1985, pp. 77-96) or trioma techniques. The technology for producing hybridomas is well known (see generally Coligan *et al.*, ed., Current Protocols in Immunology, John Wiley & Sons, New York, 1994). Hybridoma cells producing a monoclonal antibody of the invention are

detected by screening the hybridoma culture supernatants for antibodies that bind the polypeptide of interest e.g. using a standard ELISA assay.

Alternative to preparing monoclonal antibody-secreting hybridomas, a monoclonal antibody directed against a polypeptide of the invention can be identified and isolated by screening a recombinant combinatorial immunoglobulin library (e.g. an antibody phage display library) with the polypeptide of interest. Kits for generating and screening phage display libraries are commercially available (e.g. the Pharmacia Recombinant Phage Antibody System, Catalog No. 27-9400-01; end the Stratagene Su' 4P Phage Display Kit, Catalog No. 240612). Additionally examples of methods and reagents particularly amenable for use in generating and screening antibody display library can for example be found in U.S. Patent No. 5,223,409; WO 92/18619; WO 91/17271; WO 92/20791; WO 92/15679; WO 93/01288; WO 92/01047; WO 92/09690; WO 90/02809; Fuchs et al., Bio/Technology 9 (1991)1370-1372; Hay, B.N., *et al.*, Hum. Antibod. Hybridomas 3 (1992) 81-85; Huse, W.D., *et al.*, Science 246 (1989) 1275-1281; Griffiths, A.D., *et al.*, EMBO 12 (1993) 725-734.

An antibody directed against a polypeptide according to the invention (e.g. a monoclonal antibody) can be used to isolate the polypeptide by standard techniques, such as affinity chromatography or immunoprecipitation.

Moreover, such an antibody can be used to detect the polypeptide or peptide fragment (e.g. in a cellular lysate or cell supernatant) in order to evaluate the level and pattern of expression thereof. The antibodies can also be used diagnostically to monitor protein levels in tissues or body fluids (e.g. in a tissue-associated body fluid) as part of a clinical testing procedure e.g. to for example determine the efficacy of a given treatment regimen.

Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, 6-galactosidase or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin and aequorin and examples of suitable radioactive material include I, P, S or H.

Accordingly in one aspect the invention provides substantially purified antibodies or fragments thereof and non-human antibodies or fragments thereof, which antibodies or fragments specifically bind to a polypeptide comprising an amino acid sequence selected from the group consisting of the amino acid sequences of the present invention, an amino acid sequence encoded by the cDNA of the present invention, a fragment of at least 6, preferably 10 amino acid residues of an amino acid sequence of the present invention being preferably phosphorylated, an amino acid sequence which is at least 95 % identical to the amino acid sequence of the present invention (wherein the percent identity is determined using the ALIGN program of the GCG software package with a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 and an amino acid sequence which is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule consisting of the nucleic acid molecules of the present invention or a complement thereof under conditions of hybridization of 6X SSC at 45°C and washing in 0.2 X SSC, 0.1 % SDS at 65°C. In various embodiments the substantially purified antibodies of the invention or fragments thereof can be human, non-human, chimeric and/or humanized antibodies.

In another aspect the invention provides non-human antibodies or fragments thereof which antibodies or fragments specifically bind to a polypeptide comprising an amino acid sequence selected from the group consisting of the amino acid sequence of the present invention, an amino acid sequence encoded by the cDNA of the present invention, a fragment of at least 6, preferably 10 amino acid residues of the amino acid sequence of the present invention, an amino acid sequence which is at least 95 % identical to the amino acid sequence of the present invention (wherein the percent identity is determined using the ALIGN program of the GCG software package with a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 and an amino acid sequence which is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule according to the invention or a complement thereof under conditions of hybridization of 6X SSC at 45°C and washing in 0.2 X SSC, 0.1 % SDS at 65°C. Such non- human antibodies can be goat, mouse, sheep, horse, chicken, rabbit or rat antibodies. Alternatively the non-human antibodies of the invention can be chimeric and/or humanized antibodies. In addition the non human antibodies of the invention can be polyclonal antibodies or monoclonal antibodies.

In still a further aspect the invention provides monoclonal antibodies or fragments thereof which antibodies or fragments specifically bind to a peptide/polypeptide comprising an amino acid sequence selected from the group consisting of the amino acid sequences of the present invention, an amino acid sequence encoded by the cDNA of the present invention, a fragment of at least 6, preferably 10 amino acid residues of an amino acid sequence of the present invention, an amino acid sequence which is at least 95 % identical to an amino acid sequence of the present invention (wherein the percent identity is determined using the ALIGN program of the GCG software package with a PAM120 weight residue table), a gap length penalty of 12 and a gap penalty of 4 and an amino acid sequence which is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule consisting of the nucleic acid molecules of the present invention or a complement thereof under conditions of

hybridization of 6X SSC at 45°C and washing in 0.2 X SSC, 0.1 % SDS at 65°C. The monoclonal antibodies can be human, humanized, chimeric and/or non-human antibodies.

Any of the antibodies of the invention can be conjugated to a detectable substance. Non-limiting examples of detectable substances that can be conjugated to the antibodies of the invention are an enzyme, a prosthetic group, a fluorescent material, a luminescent material, a bioluminescent material and a radioactive material.

The invention also provides a kit containing an antibody of the invention conjugated to a detectable substance and instructions for use.

Still another aspect of the invention is a method of making an antibody that specifically recognizes a polypeptide or peptide fragment of the present invention, the method comprising immunizing a mammal or a vertebrate (we also produce chicken abs) with a polypeptide. The polypeptide used as an immunogen comprises an amino acid sequence selected from the group consisting of the amino acid sequence of the present invention, an amino acid sequence encoded by the cDNA of the nucleic acid molecules of the present invention, a fragment of at least 6, preferably 10 amino acid residues of the amino acid sequence of the present invention, an amino acid sequence which is at least 95 % identical to the amino acid sequence of the present invention (wherein the percent identity is determined using the ALIGN program of the GCG 3s software package with a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4 and an amino acid sequence which is encoded by a nucleic acid molecule which hybridizes to a nucleic acid molecule consisting of the nucleic acid molecules of the present invention or a complement thereof under conditions of hybridization of 6X SSC at 45°C and washing in 0.2 X SSC, 0. 1 % SDS at 65°C.

After immunization a sample is collected from the animal that contains an antibody that specifically recognizes the polypeptide. The polypeptide is preferably produced recombinantly using a non-human host cell. Optionally the antibodies can be further purified from the sample using techniques well known to a person skilled in the art.

The method can further comprise producing a monoclonal antibody-producing cell from the cells of the animal. Optionally antibodies are collected from the antibody-producing cell.

In summary, the invention provides an isolated antibody which specifically binds to a polypeptide according to the invention or to a peptide fragment according to the the invention and which has less than 10%, preferably 5 %, more preferably 2 %, 1%, 0.5 %, 0.1 % or 0.01% or even no cross reactivity with the corresponding non-phosphorylated polypeptide or corresponding non-phosphorylated peptide fragment. The cross reactivity can be determined by enzyme immuno assay or radio immuno assay as known to the expert skilled in the art and described in the application. The antibody may be a polyclonal antibody but is preferably a monoclonal antibody. The polyclonal antibody serum may be purified from cross-reacting antibodies by binding the non-phosphorylated antigen to which some antibodies might cross-react to a column and isolating the non-binding (more specific) polyclonal antibodies.

II. Nucleic acids

One aspect of the invention pertains to isolated nucleic acid molecules that encode the polypeptide or peptide fragment according to the invention, in particular the non-phosphorylable analogues thereof, or the antibody according to the invention and can therefore be used for the production of the polypeptide or antibody according to the invention or precursors thereof.

Such nucleic acid molecules comprise sequences of RNA transcripts or portions of such transcripts. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g. cDNA or genomic DNA) and RNA molecules (e.g. mRNA) and analogues of the DNA or RNA generated using nucleotide analogues. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

The invention also encompasses nucleic acids which differ from that of the nucleic acids described herein, but which produce the same phenotypic effect such as an allelic variant. These altered but phenotypically equivalent nucleic acids are referred to as "equivalent nucleic acids." This invention also encompasses nucleic acids characterized by changes in non-coding regions that do not alter the polypeptide produced therefrom when compared to the polynucleotide herein. This invention further encompasses nucleic acids which hybridize to the polynucleotides of the subject invention under conditions of moderate or high stringency. Alternatively the polynucleotides are at least 85 % or at least 90 % or more preferably greater or equal to 95 % identical as determined by a sequence alignment program when run under default parameters.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule. Preferably an "isolated" nucleic acid molecule comprises a protein-coding sequence and is free of sequences which naturally flank the coding sequence in the genomic DNA of the organism from which the nucleic acid is derived. For example in various embodiments the isolated nucleic acid molecule can contain less than about 5 kB 4 kB, 3 kB, 2 kB, 1 kB, 0.5 kB or 0.1 kB of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover an "isolated" nucleic acid molecule such as a cDNA molecule can be substantially free of other cellular material or culture medium when

produced by recombinant techniques or substantially free of chemical precursors or other chemicals when chemically synthesized.

A nucleic acid molecule of the present invention can be isolated using standard molecular biology techniques. A nucleic acid molecule of the present invention also encompasses the nucleic acid molecules which can be isolated using standard hybridization and cloning techniques (e.g. as described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

The nucleic acid molecule according to the invention can be amplified using cDNA, mRNA or genomic DNA as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid amplified in this manner can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore oligonucleotides corresponding to all or a portion of the nucleic acid molecule according to the invention can be prepared by standard synthetic techniques e.g. using an automated DNA synthesizer. In another preferred embodiment an isolated nucleic acid molecule of the invention comprises a nucleotide sequence of a RNA transcript according to the invention or a complement of said sequence. A nucleic acid molecule which is complementary to a given nucleotide sequence is one which is sufficiently complementary to the given nucleotide sequence that it can hybridize to the given nucleotide sequence thereby forming a stable duplex.

Moreover a nucleic acid molecule of the invention can comprise only a portion of the nucleotide sequence (RNA or cDNA) of a RNA transcript or a complement of said sequence. Such nucleic acids can for example be used as a probe or primer. The probe/primer is typically used as one or more substantially purified oligonucleotides. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 7, preferably about 15, more

preferably about 25, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, or 400 or more consecutive nucleotides of the nucleic acid molecule according to the invention.

Probes based on the sequence of the nucleic acid sequence according to the invention can be used to detect transcripts or genomic sequences of the nucleic acid molecule according to the invention. The probe comprises a label group attached thereto e.g. a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Such probes can generally be used as part of a diagnostic test kit for identifying cells or tissues which mix-express the protein, such as by measuring levels of a nucleic acid molecule encoding the protein in a sample of cells from a subject e.g. detecting mRNA levels or determining whether a gene encoding the protein has been mutated or deleted.

The invention further encompasses nucleic acid molecules that differ due to degeneracy of the genetic code from the nucleotide sequence of nucleic acids encoding a protein which corresponds to the nucleic acid molecule according to the invention and thus encode the same protein.

It will be appreciated by a person skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequence can exist within a population (e.g. the human population). Such genetic polymorphisms can exist among individuals within a population due to natural allelic variation. An allele is one of a group of genes which occur alternatively at a given genetic locus. In addition it will be appreciated that DNA polymorphisms that affect RNA expression levels can also exist that may affect the overall expression level of that gene (e.g. by affecting regulation or degradation). As used herein the phrase "allelic variant" refers to a nucleotide sequence which occurs at a given locus or to a polypeptide encoded by the nucleotide sequence.

As used herein the terms "gene" and "recombinant gene" refer to nucleic acid molecules comprising an open reading frame encoding a polypeptide according to the invention. Such natural allelic variations can typically result in 0.1-0.5 % variance in the nucleotide sequence of a given gene. Alternative alleles can be identified by sequencing the gene of interest in a number of different individuals. This can be readily carried out by using hybridization probes to identify the same genetic locus in a variety of individuals. Any and all such nucleotide variations and resulting amino acid polymorphisms or variations that are the result of natural allelic variation and that do not alter the functional activity are intended to be within the scope of the invention.

As used herein the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 75 % (80 %, 85 % preferably 90 %) identical to each other typically remain hybridized to each other. Such stringent conditions are known to persons skilled in the art and can be found in sections 6.3.1 -6.3.6 of Current Protocols in Molecular Biology, John Wiley & Sons, N.Y.(1989). A preferred non-limiting example of stringent hybridization conditions for annealing two single-stranded DNA each of which is at least about 100 bases in length and/or for annealing a single-stranded DNA and a single-stranded RNA each of which is at least about 100 bases in length, means hybridization at 65°C in a hybridization buffer consisting of 250 mmol/l sodium phosphate buffer pH 7.2, 7 % (w/v) SDS, 1 % (w/v) BSA, 1 mmol/l EDTA and 0.1 mg/ml single-stranded salmon sperm DNA. A final wash is performed at 65°C in 125 mmol/l sodium phosphate buffer pH 7.2, 1 mmol/l EDTA and 1 % (w/v) SDS. Further preferred hybridization conditions are taught in Lockhart, D.J., *et al.*, Nat. Biotechnol. 14 (1996) 1675-1680; Breslauer, K.J., *et al.*, Proc. Natl. Acad. Sci. USA 83 (1986) 3746-3750; van Ness, J., and Chen, L., Nucleic Acids Res. 19 (1991) 5143-5151; McGraw, R.A., *et al.*, BioTechniques 8 (1990) 674-678; and Milner, N., *et al.*, Nat. Biotechnol. 15 (1997) 537-541, all expressly incorporated by reference. In addition to naturally-occurring allelic variants of the nucleic acid sequence according

to the invention that can exist in the population, the skilled artisan will further appreciate that sequence changes can be introduced by mutation thereby leading to changes in the amino acid sequence of the encoded protein, without altering the biological activity of the protein encoded thereby. For example one can make nucleotide substitutions leading to amino acid substitutions at "non-essential" amino acid residues. A "non essential" amino acid residue is a residue that can be altered from the wild-type sequence without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example amino acid residues that are not conserved or only semi-conserved among homologues of various species may be non essential for activity and thus would be likely targets for alteration. Alternatively amino acid residues that are conserved among the homologues of various species (e.g. murine and human) may be essential for activity and thus would not be likely targets for alteration. Accordingly another aspect of the invention pertains to nucleic acid molecules encoding a polypeptide of the invention that contain changes in amino acid residues that are not essential for activity. Such polypeptides differ in amino acid sequence from the naturally-occurring proteins encoded by the nucleic acid sequence according to the invention, yet retain biological activity. In one embodiment such a protein has an amino acid sequence that is at least about 40 % identical, 50 %, 60 %, 70 %, 80 %, 90 %, 95 %, or 98 % identical to the amino acid sequence of one of the proteins according to the invention. An isolated nucleic acid molecule encoding a variant protein can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of nucleic acids of the invention such that one or more amino acid residue substitutions, additions or deletions are introduced into the encoded protein.

Mutations can be introduced by standard techniques such as site-directed mutagenesis and PCR-mediated mutagenesis. Conservative amino acid substitutions are preferably made at one or more predicted non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino

acid residues having similar side chains have been deemed in the art. These families include amino acids with basic side chains (e.g. lysine, arginine, histidine) acidic side chains (e.g. aspartic acid, glutamic acid) uncharged polar side chains (e.g. glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine) non-polar side chains (e.g. alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan) beta-branched side chains (e.g. threonine, valine, isoleucine) and aromatic side chains (e.g. tyrosine, phenylalanine, tryptophan, histidine). Alternatively mutations can be introduced randomly along all or part of the coding sequence such as by saturation mutagenesis and the resultant mutants can be screened for biological activity to identify mutants that retain activity. Following mutagenesis the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

III. Recombinant Expression Vectors and Host Cells

Another aspect of the invention pertains to vectors preferably expression vectors, containing a nucleic acid sequence encoding a polypeptide according to the invention (or a portion of such a polypeptide as the peptide fragment).

As used herein the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid" which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g. bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g. non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell and thereby are

replicated along with the host genome. Moreover, certain vectors namely expression vectors are capable of directing the expression of genes to which they are operably linked. In general expression vectors of utility in recombinant DNA techniques are often in the form of plasmids (vectors). However, the invention is intended to include such other forms of expression vectors such as viral vectors (e. g. replication defective retroviruses, adenoviruses and adeno-associated viruses) which serve equivalent functions. The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell. This means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression which is operably linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g. in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g. polyadenylation signals). Such regulatory sequences are described for example in Goeddel, Methods in Enzymology: Gene Expression Technology, Vol. 185, Academic Press, San Diego, CA, 1991. Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g. tissue-specific regulatory sequences). It will be appreciated by persons skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, and the like. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein.

The recombinant expression vectors of the invention can be designed for expression of a polypeptide according to the invention in prokaryotic (e.g. E. coli) or eukaryotic cells (end insect cells using baculovirus expression vectors, yeast cells or mammalian cells). Suitable host cells are discussed further in Goeddel, supra. Alternatively the recombinant expression vector can be transcribed and translated in vitro for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in E. coli with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein usually to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often in fusion expression vectors a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes and their cognate recognition sequences include Factor Xa, thrombin and enterokinase.

Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B., and Johnson, K.S., Gene 67 (1988) 31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Phannacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein or protein A respectively to the target recombinant protein. Examples of suitable inducible non-fusion E. coli expression vectors include pTrc (Amann, E., *et al.*, Gene 69 (1988) 301-315) and pET 1 Id (Studier *et al.*, In: Gene Expression Technology: Methods in Enzymology, Vol. 85, Academic Press, San Diego, CA, 1991, pp. 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 1 Id vector relies on transcription

from a T7 gulO-lac fusion promoter mediated by a co-expressed viral RNA polymerase (T7 gal). This viral polymerase is supplied by host strains BL21(DE3) or HMS 174(DE3) from a resident prophage harboring a T7 gal gene under the transcriptional control of the lacUV promoter.

One strategy to maximize recombinant protein expression in E. coli is to express the protein in a host bacterium with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, In: Gene Expression Technology: Methods in Enzymology, Vol. 185, Academic Pres, San Diego, CA, 1990, pp. 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in E. coli (Wada, K., *et al.*, Nucleic Acids Res. 20 (1992) 2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques. In another embodiment the expression vector is a yeast expression vector.

Examples of vectors for expression in yeast S. cerevisiae include pYepSecl (Baldari, C. *et al.*, EMBO J. 6 (1987) 229-234), pMFa (Kurjan, J. and Herskowitz, I., Cell 30 (1982) 933-943), pJRY88 (Schultz, L.D. *et al.*, Gene 54 (1987) 113-123), pYES2 (Invitrogen Corporation, San Diego, CA) and pPicZ (Invitrogen Corp, San Diego, CA).

Alternatively the expression vector is a baculovirus expression vector. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g. Sf9 cells) include the pAc series (Smith, G.E., et al., Mol. Cell Biol. 3 (1983) 2156-2165) and the pVL series (Luckow, V.A., and Summers, M.D., Virology 170 (1989) 31-39).

In yet another embodiment the protein according to the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B., Nature 329 (1987) 840-842) and

pMT2NOPC (Kaufman, R.J., et al., EMBO J. 6 (1987) 187-193). When used in mammalian cells the expression vector's control functions are often provided by viral regulatory elements.

For example commonly used promoters are derived from polyoma, adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook *et al.*, supra.

In another embodiment the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (e.g. tissue-specific regulatory elements are used to express the nucleic acid). Tissue specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert, C.A. *et al.*, Genes Dev. 1 (1987) 268-277), lymphoid-specific promoters (Calame, K. and Eaton, S., Adv. Immunol. 43 (1988) 235-275) in particular promoters of T cell receptors (Winoto, A., and Baltimore, D., EMBO J. 8 (1989) 729-733) and immunoglobulins (Banerji, J., *et al.*, Cell 33 (1983) 729-740; Queen, C. and Baltimore, D., Cell 33 (1983) 741-748), neuron-specific promoters (e.g. the neurofilament promoter; Byrne, G.W., and Ruddle, F.H., Proc. Natl. Acad. Sci. USA 86 (1989) 5473-5477), pancreas-specific promoters (Edlund, T., et al., Science 230 (1985) 912-916) and mammary gland-specific promoters (e.g. milk whey promoter, U.S. Patent No. 4,873,316 and EP-A 0 264 166). Developmentally regulated promoters are also encompassed for example the murine box promoters (Kessel, M., and Gruss, P., Science 249 (1990) 374-379) and the alpha-fetoprotein promoter (Camper, S.A., and Tilghman, S.M., Genes Dev. 3 (1989) 537-546).

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential

progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences such progeny may not in fact be identical to the parent cell but are still included within the scope of the term as used herein. A host cell can be any prokaryotic (e.g. E. coli) or eukaryotic cell (e.g. insect cells, yeast or mammalian cells).

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized; techniques for introducing foreign nucleic acid into a host cell, including calcium phosphate or calcium chloride co- precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, *et al.* (supra) and other laboratory manuals.

For stable transfection of mammalian cells it is known that depending upon the expression vector and transfection technique used only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants a gene that encodes a "selectable marker" (SM) gene (e.g. for resistance to antibiotics) is generally introduced into the host cells along with the gene of interest.

Preferred SM genes include those which confer resistance to drugs such as G418, hygromycin and methotrexate. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g. cells that have incorporated the SM gene will survive while the other cells die).

A host cell of the invention such as a prokaryotic or eukaryotic host cell in culture can be used to produce a polypeptide according to the invention. Accordingly the invention further provides methods for producing a polypeptide according to the invention using the host cells of the invention. In one embodiment the method comprises culturing the host cell of invention (into which a recombinant expression

vector encoding a polypeptide of the invention has been introduced) in a suitable medium such that the polypeptide according to the invention is produced. In another embodiment the method further comprises isolating the polypeptide from the medium or the host cell. The host cells of the invention can also be used to produce nonhuman transgenic animals. For example in one embodiment a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which a sequences encoding a polypeptide according to the invention have been introduced. Such host cells can then be used to create non-human transgenic animals in which exogenous sequences encoding the polypeptide according to the invention have been introduced into their genome or homologous recombinant animals in which endogenous gene(s) encoding a polypeptide according to the invention have been altered.

IV. Predictive Medicine

The present invention pertains to the field of predictive medicine in which diagnostic assays, prognostic assays, pharmacogenomics and monitoring clinical trials are used for prognostic (predictive) purposes to thereby treat an individual prophylactically. Accordingly one aspect of the present invention relates to diagnostic assays for determining the level of the phosphorylated protein according to the invention. Such assays can be used for prognostic or predictive purposes to thereby prophylactically treat an individual prior to the metastasis of the cancer.

A particularly preferred embodiment of the invention in this context , is a method for the determination of the amount or presence of a polypeptide according to the invention or a peptide fragment according to the invention in a sample comprising the steps of

a) providing a sample suspected to contain the polypeptide or the peptide fragment,

b) incubating the sample in the presence of an antibody according to the invention, and

c) determining the binding product between the polypeptide and the antibody thereby

concluding that the polypeptide or peptide fragment is present or thereby deriving the amount of the polypeptide or the peptide fragment.

Another preferred embodiment is a method of determining whether or not a human cancer cell containing patient sample has potential for tumor progression, the method comprising comparing:

a) the level of expression a polypeptide according to the invention or a peptide fragment according to the invention in the patient sample, and
b) the normal level of expression the polypeptide or the peptide fragment in a sample from a control subject not afflicted with cancer,
and a significant difference between the level of expression of the polypeptide or the peptide fragment in the patient sample and the normal level of the polypeptide or the peptide fragment in the sample from a control subject not afflicted with cancer is an indication that the patient sample has potential for tumor progression.

In a preferred embodiment, the polypeptide according to the invention or the peptide fragment according to the invention are used for the determination of the potential of a human cancer cell for tumor progression.

In both methods, the sample is a tissue sample, blood or blood derived cells, primary cell cultures from patients, stool, lymph or a tissue-associated fluid or urine.

The presence of said polypeptide or peptide fragment is preferably detected using a reagent which specifically binds with said polypeptide or peptide fragment, preferably the reagent is selected from the group consisting of an antibody, an antibody derivative, and an antibody fragment. This is described in more detail below.

In an alternative embodiment the ratio of phosphorylated versus unphosphorylated polypeptide is determined for each sample

An exemplary method for detecting the presence or absence of a phosphorylated polypeptide according to the invention in a biological sample involves obtaining a biological sample (e.g. a biopsy) from a test subject and contacting the biological

sample with a compound or an agent capable of detecting the polypeptide. The detection methods of the invention can thus be used to detect protein for example in a biological sample in vitro as well as in vivo. In vitro techniques for detection of a polypeptide according to the invention include enzyme linked immunosorbent assays (ELISAs), Western blots, immunoprecipitations, immunohistochemistry and immunofluorescence. Furthermore in vivo techniques for detection of a polypeptide according to the invention include introducing into a subject a labelled antibody directed against the polypeptide. For example the antibody can be labelled with a radioactive marker whose presence and location in a subject can be detected by standard imaging techniques.

A general principle of such diagnostic and prognostic assays involves preparing a sample or reaction mixture that may contain a protein or polynucleotide and a probe, under appropriate conditions and for a time sufficient to allow the protein or nucleotide and probe to interact and bind, thus forming a complex that can be removed and/or detected in the reaction mixture. These assays can be conducted in a variety of ways.

For example one method to conduct such an assay would involve anchoring the protein or nucleotide on the one hand or probe on the other onto a solid phase supports also referred to as a substrate and detecting complexes comprising the target nucleic acid molecule or protein and the probe anchored on the solid phase at the end of the reaction. In one embodiment of such a method a sample from a subject which is to be assayed for presence and/or concentration of the proteins or nucleotides encoded by the nucleic acid molecule can be anchored onto a carrier or solid phase support. In another embodiment the reverse situation is possible in which the probe can be anchored to a solid phase and a sample from a subject can be allowed to react as an unanchored component of the assay.

There are many established methods for anchoring assay components to a solid phase. These include without limitation the protein or nucleic acid molecules according to the invention or probe molecules which are immobilized through conjugation of biotin and streptavidin. Such biotinylated assay components can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (e.g. biotinylation kit, Pierce Chemicals, Rockford, IL) and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). In certain embodiments the surfaces with immobilized assay components can be prepared in advance and stored.

Other suitable carriers or solid phase supports for such assays include any material capable of binding the class of molecule to which the protein according to the invention or nucleotide or probe belongs. Well-known supports or carriers include but are not limited to glass, polystyrene, nylon, polypropylene, nylon, polyethylene, dextran, amylases, natural and modified celluloses, polyacrylamides, gabbros and magnetite.

In order to conduct assays with the above-mentioned approaches the non immobilized component is added to the solid phase upon which the second component is anchored. After the reaction is complete uncomplexed components may be removed (e.g. by washing) under conditions such that any complexes formed will remain immobilized upon the solid phase. The detection of complexes comprising the protein of the invention and the probe anchored to the solid phase can be accomplished in a number of methods outlined herein. In a preferred embodiment the probe when it is the unanchored assay component can be labelled for the purpose of detection and readout of the assay, either directly or indirectly with detectable labels discussed herein and which are well-known to one skilled in the art.

It is also possible to directly detect complexes comprising a protein of the invention and the probe without further manipulation or labelling of either component (the

protein of the invention or nucleotide or the probe) for example by utilizing the technique of fluorescence energy transfer (see for example U.S. Patent No. 5,631,169 and U.S. Patent No. 4,868,103). A fluorophore label on the first 'donor' molecule is selected such that upon excitation with incident light of appropriate wavelength its emitted fluorescent energy will be absorbed by a fluorescent label on a second 'acceptor' molecule, which in turn is able to fluoresce due to the absorbed energy. Alternately the 'donor' protein molecule may simply utilize the natural fluorescent energy of tryptophan residues. Labels are chosen that emit different wavelengths of light such that the 'acceptor' molecule label may be differentiated from that of the 'donor'. Since the efficiency of energy transfer between the labels is related to the distance separating the molecules, spatial relationships between the molecules can be assessed. In a situation in which binding occurs between the molecules, the fluorescent emission of the 'acceptor' molecule label in the assay should be maximal. A PET binding event can be conveniently measured through standard fluorometric detection means well known in the art (e.g. using a fluorimeter).

In another embodiment determination of the ability of a probe to recognize a protein can be accomplished without labelling either assay component (probe) by utilizing a technology such as real time Biomolecular Interaction Analysis (BIA) (see e.g. Sjolander, S. and Urbaniczky, C., Anal. Chem. 63 (1991) 2338-2345 and Szabo, A., *et al.*, Curr. Opin. Struct. Biol. 5 (1995) 699-705). As used herein "BIA" or "surface plasmon resonance" is a technology for studying biospecific interactions in real-time without labelling any of the interactants (e.g. BIAcore). Changes in the mass at the binding surface (indicative of a binding event) result in alterations of the refractive index of light near the surface (the optical phenomenon of surface plasmon resonance (SPR)) resulting in a detectable which can be used as an indication of real-time reactions between biological molecules.

Alternatively in another embodiment, analogous diagnostic and prognostic assays can be conducted with the protein of the invention and the probe as solutes in a liquid

phase. In such an assay complexes comprising the protein of the invention and the probe are separated from uncomplexed components by any of a number of standard techniques, including but not limited to differential centrifugation, chromatography, electrophoresis and immunoprecipitation. In differential centrifugation such complexes may be separated from uncomplexed assay components through a series of centrifugal steps due to the different sedimentation equilibria of complexes based on their different sizes and densities (see for example Rivas, G., and Minton, A.P., Trends Biochem Sci. 18 (1993) 284-287). Standard chromatographic techniques may also be utilized to separate such complexes from uncomplexed components. For example gel filtration chromatography separates molecules based on size and through the utilization of an appropriate gel filtration resin in a column format for example the relatively larger complexes may be separated from the relatively smaller uncomplexed components. Similarly the different charge properties of such complexes as compared to the uncomplexed components may be exploited to differentiate the complexes from uncomplexed components for example through the utilization of ion-exchange chromatography resins. Such resins and chromatographic techniques are well known to a person skilled in the art (see e.g. Heegaard, N.H., J. Mol. Recognit. 11 (1998) 141-148; Hage, D.S., and Tweed, S.A., J. Chromatogr. B. Biomed. Sci. Appl. 699 (1997) 499-525). Gel electrophoresis may also be employed to separate such complexes from unbound components (see e.g., Ausubel et al., ed., Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1987 1999). In this technique protein complexes are separated based on size or charge for example. In order to maintain the binding interaction during the electrophoretic process non-denaturing gel matrix materials and conditions in the absence of reducing agent are typically preferred. Appropriate conditions to the particular assay and components thereof will be well known to one skilled in the art.

In another embodiment of the present invention a polypeptide of the invention is detected. A preferred agent for detecting a polypeptide of the invention is an antibody capable of binding to a polypeptide according to the invention preferably an

antibody with a detectable label. Antibodies can be polyclonal or more preferably monoclonal. An intact antibody or a fragment thereof (e.g. Fab or $F(ab')_2$) can be used. The term "labelled" with regard to the probe or antibody is intended to encompass direct labelling of the probe or antibody by coupling (i. e. physically linking) a detectable substance to the probe or antibody as well as indirect labelling of the probe or antibody by reactivity with another reagent that is directly labelled. Examples of indirect labelling include detection of a primary antibody using a fluorescently labelled secondary antibody and end-labelling of a DNA probe with biotin such that it can be detected with fluorescently labelled streptavidin.

Proteins from cells can be isolated using techniques that are well-known to a person skilled in the art. The protein isolation methods employed can for example be such as those described in Harlow and Lane, Antibodies: Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1988). A variety of formats can be employed to determine whether a sample contains a protein that binds to a given antibody. Examples of such formats include, but are not limited to, enzyme immunoassay (EIA), radioimmunoassay (RIA), Western blot analysis, immunohisto-chemistry and enzyme linked immunoabsorbent assay (ELISA).

A skilled artisan can readily adapt known protein/antibody detection methods for use in determining whether cancer cells express a nucleic acid molecule of the present invention and modifies the expressed protein.

In one format antibodies or antibody fragments can be used in methods such as Western blots, immunohistochemistry or immunofluorescence techniques to detect the expressed proteins. In such uses it is generally preferable to immobilize either the antibody, proteins or cells containing proteins on a solid support. Well-known supports or carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, gabbros and magnetite.

One skilled in the art will know many other suitable carriers for binding antibody or antigen and will be able to adapt such support for use with the present invention. For example protein isolated from cancer cells can be run on a polyacrylamide gel electrophoresis and immobilized onto a solid phase support such as nitrocellulose or PVDF. The support can then be washed with suitable buffers followed by treatment with the detectably labelled antibody. The solid phase support can then be washed with the buffer a second time to remove unbound antibody. The amount of bound label on the solid support can then be detected by conventional means.

The invention also encompasses kits for detecting the presence of a polypeptide according to the invention in a biological sample (e.g. a tissue-associated body fluid). Such kits can be used to determine if a subject is suffering from or is at increased risk of developing metastatic cancer. For example the kit can comprise a labelled compound or agent capable of detecting a polypeptide or an mRNA encoding a polypeptide according to the invention in a biological sample and means for determining the amount of the polypeptide or mRNA in the sample (e.g. an antibody which binds the polypeptide or an oligonucleotide probe which binds to DNA or mRNA encoding the polypeptide). Kits can also include instructions for interpreting the results obtained using the kit.

Therefore, in an embodiment of the invention a kit for the detection or determination of the amount of the polypeptide according to the invention or the peptide fragment according to the invention in a biological sample is provided which comprises:
(a) an antibody according to the invention and
(b) a label for qualitatively or quantitatively detecting an immunoconjugate of the antibody and the polypeptide or the peptide fragment. This is described in more detail below.

For antibody-based kits the kit can comprise for example: (1) a first antibody (e.g. attached to a solid support) which binds to a polypeptide according to the invention

and optionally (2) a second different antibody which binds to either the polypeptide or the first antibody and is conjugated to a detectable label. For oligonucleotide-based kits the kit can comprise for example (1) an oligonucleotide e.g. a detectably labelled oligonucleotide which hybridizes to a nucleic acid sequence encoding a polypeptide according to the invention or (2) a pair of primers useful for amplifying the nucleic acid molecule according to the invention. The kit can also comprise e.g. a buffering agent, a preservative or a protein stabilizing agent. The kit can further comprise components necessary for detecting the detectable label (e.g. an enzyme or a substrate). The kit can also contain a control sample or a series of control samples which can be assayed and compared to the test sample. Each component of the kit can be enclosed within an individual container and all of the various containers can be within a single package, along with instructions for interpreting the results of the assays performed using the kit.

V. Pharmaceutical Compositions

In a preferred embodiment of the invention, agents which modulate expression or activity of a polypeptide according to the invention are provided for use in medicine, i.e. a non-phosphorylable polypeptide according to the invention, a non-phosphorylable peptide fragment according to the invention, a nucleic acid molecule according to the invention or an antibody according to the invention is provided for use in medicine. Further, a pharmaceutical composition comprising a non-phosphorylable polypeptide according to the invention, a non-phosphorylable peptide fragment according to the invention, a nucleic acid molecule according to the invention or an antibody according to the invention together with a pharmaceutically acceptable carrier is provided. In another embodiment, the non-phosphorylable polypeptide according to the invention, the non-phosphorylable peptide fragment according to the invention, the nucleic acid molecule according to the invention or

the antibody according to the invention is used for the preparation of a pharmaceutical composition for the treatment of hyperproliferative disease, preferably cancer.

In another, substances may be used in medicine that inhibit the tyrosine modification of p27.

The invention includes methods for preparing pharmaceutical compositions for modulating the expression or activity of a polypeptide according to the invention. Such methods comprise formulating a pharmaceutically acceptable carrier with an agent which modulates expression or activity of a polypeptide or nucleic acid according to the invention. Such compositions can further include additional active agents. Thus the invention further includes methods for preparing a pharmaceutical composition by formulating a pharmaceutically acceptable carrier with an agent which modulates expression or activity of a polypeptide according to the invention and one or more additional active compounds. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

The invention also provides methods (also referred to herein as "screening assays") for identifying modulators, i. e. candidate or test compounds or agents (e.g. peptides, peptidomimetics, peptoids, small molecules or other drugs) which (a) bind to the polypeptide according to the invention or (b) have a modulatory (e.g. stimulatory or inhibitory) effect on the activity of the nucleic acid molecule or more specifically (c)

have a modulatory effect on the interactions of a protein with one or more of its natural substrates (e.g. peptide, protein, hormone, co-factor or nucleic acid). Such assays typically comprise a reaction between the protein of the invention and one or more assay components. The other components may be either the test compound itself or a combination of test compound and a natural binding partner of the protein according to the invention.

Therefore, a method of deriving a candidate agent or test compound is provided, said method comprising:

(a) contacting a sample containing cancer cells, with said candidate agent or test compound;
(b) determining the level of expression of the polypeptide according to the invention or a peptide fragment according to the invention in the sample contacted with the candidate agent or test compound and determining the level of expression of the polypeptide in a sample not contacted with the candidate agent or test compound;
(c) observing the effect of the candidate agent or test compound by comparing the level of expression of the polypeptide or the peptide fragment in the sample contacted with the candidate agent or test compound and the level of the polypeptide or the peptide fragment in the sample not contacted with the candidate agent or test compound,
(d) deriving said agent from said observed effect,
wherein an at least 1.5 fold difference or a less than 0.75 fold difference between the level of expression of the polypeptide or the peptide fragment in the sample contacted with the candidate agent or test compound and the level of expression of the polypeptide or the peptide fragment in the sample not contacted with the candidate agent or test compound is an indication of an effect of the candidate agent or test compound. Preferably, said candidate agent or test compound is a candidate inhibitory agent or a candidate enhancing agent.

The test compounds of the present invention may be obtained from any available source including systematic libraries of natural and/or synthetic compounds. Test compounds may also be obtained by any of the numerous approaches in

combinatorial library methods known in the art, including biological libraries, peptoid libraries (libraries of molecules having the functionalities of peptides but with a novel non peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive, see e.g. Zuckermann, R.N., *et al.*, J. Med. Chem. 37 (1994) 2678-2685), spatially addressable parallel solid phase or solution phase libraries, synthetic library methods requiring deconvolution, the 'one-bead one-compound' library method and synthetic library methods using affinity chromatography selection. The biological library and peptoid library approaches are limited to peptide libraries while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S., Anticancer Drug Des. 12 (1997) 145-167).

Examples of methods for the synthesis of molecular libraries can be found in the art for example in DeWitt, S.H., et al., Proc. Natl. Acad. Sci. USA 90 (1993) 6909-6913; Erb, E., *et al.*, Proc. Natl. Acad. Sci. USA 91 (1994) 11422-11426; Zuckermann, R.N., *et al.*, J. Med. Chem. 37 (1994) 2678-2685; Cho, C.Y., *et al.*, Science 261 (1993) 1303-1305; Carrell *et al.*, Angew. Chem. Int. Ed. Erg. 33 (1994) 2059; Carell *et al.*, Angew. Chem. Int. Ed. Engl. 33 (1994) 2061 and in Gallop, M.A., *et al.*, J. Med. Chem. 37 (1994) 1233-1251.

Libraries of compounds may be presented in solution (e.g. Houghten, R.A., *et al.*, Biotechniques 13 (1992) 412-421) or on beads (Lam, K.S., *et al.*, Nature 354 (1991) 82-84), chips (Fodor, S.P., *et al.*, Nature 364 (1993) 555-556), bacteria and/or spores, (US 5,223,409), plasmids (Cull, M.G., *et al.*, Proc. Natl. Acad. Sci. USA 89 (1992) 1865-1869) or on phage (Scott, J.K., and Smith, G.P., Science 249 (1990) 386-390; Devlin, J.J., *et al.*, Science 249 (1990) 404-406; Cwirla, S.E., *et al.*, Proc. Natl. Acad. Sci. 87 (1990) 6378-6382; Felici, F., *et al.*, Mol. Biol. 222 (1991) 301-310; US 5,223,409). In one embodiment the invention provides assays for screening candidate or test compounds which are substrates of the protein of the invention or biologically active portion thereof. In another embodiment the invention provides assays for

screening candidate or test compounds which bind to a protein according to the invention or biologically active portion thereof.

Determining the ability of the test compound to directly bind to a polypeptide of the invention can for example be accomplished by coupling the compound with a radioisotope or enzymatic label such that binding of the compound to the protein can be determined by detecting the protein compound in a labelled complex. For example compounds (e.g. substrates of the protein of the invention) can be labelled with 25I, 35S, 14C or 3H either directly or indirectly and the radioisotope detected by direct counting of radioemission or by scintillation counting. Alternatively assay components can be enzymatically labelled with for example horseradish peroxidase, alkaline phosphatase or luciferase and the enzymatic label detected by determination of conversion of an appropriate s substrate to product.

In another embodiment the invention provides assays for screening candidate or test compounds which modulate the activity of the polypeptide of the invetion or a biologically active portion thereof. In all likelihood the protein can in vivo interact with one or more molecules such as but not limited to peptides, proteins, hormones, cofactors and nucleic acids. For the purposes of this discussion such cellular and extracellular molecules are referred to herein as "binding partners" or protein "substrate".

One necessary embodiment of the invention in order to facilitate such screening is the use of the protein according to the invention to identify its natural in vivo binding partners. There are many ways to accomplish this which are known to a person skilled in the art. One example is the use of the protein of the invention as "bait protein" in a two-hybrid assay or three-hybrid assay (see e.g. U.S. Patent No. 5,283,317; Zervos, A.S., et al., Cell 72 (1993) 223-232; Madura, K., *et al.*, J. Biol. Chem. 268 (1993) 12046-12054; Bartel, P., *et al.*, Biotechniques 14 (1993) 920-924; Iwabuchi, K., *et al.*, Oncogene 8 (1993) 1693-1696; WO 94/10300) in order to

identify other proteins which bind to or interact with the protein (binding partners) and therefore are possibly involved in the natural function of the protein. Such protein binding partners are also likely to be involved in the propagation of signals by the protein or downstream elements of a gene-mediated signalling pathway. Alternatively such protein binding partners may also be found to be inhibitors of the protein.

The two-hybrid system is based on the modular nature of most transcription factors which consist of separable DNA-binding and activation domains. Briefly the assay utilizes two different DNA constructs. In one construct the gene that encodes a protein fused to a gene encoding the DNA binding domain of a known transcription factor (e.g. GAL-4). In the other construct a DNA sequence from a library of DNA sequences that encodes an unidentified protein ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. If the "bait" and the "prey" proteins are able to interact in vivo forming a gene dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (e.g. LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be readily detected and cell clones containing the functional transcription factor can be isolated and used to obtain the cloned gene which encodes the protein which interacts with the protein of the invention.

In a further embodiment assays may be devised through the use of the invention for the purpose of identifying compounds which modulate (e.g. affect either positively or negatively) interactions between the protein of the invention and its substrates and/or binding partners. Such compounds can include, but are not limited to, molecules such as antibodies, peptides, hormones, oligonucleotides, nucleic acids and analogues thereof.

Such compounds may also be obtained from any available source, including systematic libraries of natural and/or synthetic compounds. The preferred assay components for use in this embodiment is the protein of the invention, the known binding partner and/or substrate of same and the test compound. Test compounds can be supplied from any source.

The basic principle of the assay systems used to identify compounds that interfere with the interaction between the protein and its binding partner involves preparing a reaction mixture containing the protein and its binding partner under conditions and for a time sufficient to allow the two products to interact and bind thus forming a complex. In order to test an agent for inhibitory activity, the reaction mixture is prepared in the presence and absence of the test compound. The test compound can be initially included in the reaction mixture or can be added at a time subsequent to the addition of the protein and its binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the protein and its binding partner is then detected. The formation of a complex in the control reaction but less or no such formation in the reaction mixture containing the test compound indicates that the compound interferes with the interaction of the protein of the invention and its binding partner. Conversely the formation of more complex in the presence of compound than in the control reaction indicates that the compound may enhance interaction of the protein and its binding partner.

The assay for compounds that interfere with the interaction of the protein with its binding partner may be conducted in a heterogeneous or homogeneous format.

Heterogeneous assays involve anchoring either the protein of the invention or its binding partner onto a solid phase and detecting complexes anchored to the solid phase at the end of the reaction. In homogeneous assays the entire reaction is carried out in a liquid phase. In either approach the order of addition of reactants can be

varied to obtain different information about the compounds being tested. For example test compounds that interfere with the interaction between the protein of the invention and the binding partners (e.g. by competition) can be identified by conducting the reaction in the presence of the test substance, i. e. by adding the test substance to the reaction mixture prior to or simultaneously with the protein and its interactive binding partner.

Alternatively test compounds that disrupt preformed complexes e.g. compounds with higher binding constants that displace one of the components from the complex can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are briefly described below.

In a heterogeneous assay system either the protein of the invention or its binding partner is anchored onto a solid surface or matrix while the other corresponding non-anchored component may be labelled, either directly or indirectly. In practice microtitre plates are often utilized for this approach. The anchored species can be immobilized by a number of methods either non-covalent or covalent that are typically well known to one who practices the art. Non-covalent attachment can often be accomplished simply by coating the solid surface with a solution of the protein or its binding partner and drying.

Alternatively an immobilized antibody specific for the assay component to be anchored can be used for this purpose. Such surfaces can often be prepared in advance and stored.

In related embodiments a fusion protein can be provided which adds a domain that allows one or both of the assay components to be anchored to a matrix. For example glutathione-S-transferase/protein of the invention fusion proteins or glutathione-S-transferase/binding partner can be adsorbed onto glutathione Sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates which are then

combined with the test compound or the test compound and either the non-adsorbed protein or its binding partner and the mixture incubated under conditions conducive to complex formation (e.g. physiological conditions). Following incubation the beads or microtitre plate wells are washed to remove any unbound assay components, the immobilized complex assessed either directly or indirectly for example as described above. Alternatively the complexes can be dissociated from the matrix and the level of protein binding or activity determined using standard techniques. Other techniques for immobilizing proteins on matrices can also be used in the screening assays of the invention. For example either a protein of the invention or its binding partner can be immobilized utilizing conjugation of biotin and streptavidin. Biotinylated protein or target molecules can be prepared from biotin-NHS (N-hydroxy succinimide) using techniques known in the art (e.g. biotinylation kit, Pierce Chemicals, Rockford, IL) and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). In certain embodiments the protein-immobilized surfaces can be prepared in as advance and stored.

In order to conduct the assay the corresponding partner of the immobilized assay component is exposed to the coated surface with or without the test compound. After the reaction is complete unreacted assay components are removed (e.g. by washing) and any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways.

Where the non-immobilized component is pre-labelled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized component is not pre-labelled, an indirect label can be used to detect complexes anchored on the surface e.g. using a labelled antibody specific for the initially non-immobilized species (the antibody in turn can be directly labelled or indirectly labelled). Depending upon the order of addition of reaction components

test compounds which modulate (inhibit or enhance) complex formation or which disrupt preformed complexes can be detected.

In an alternate embodiment of the invention a homogeneous assay may be used. This is typically a reaction analogous to those mentioned above which is conducted in a liquid phase in the presence or absence of the test compound. The formed complexes are then separated from unreacted components and the amount of complex formed is determined. As mentioned for heterogeneous assay systems the order of addition of reactants to the liquid phase can yield information about which test compounds modulate (inhibit or enhance) complex formation and which disrupt preformed complexes. In such a homogeneous assay the reaction products may be separated from unreacted assay components by any of a number of standard techniques including but not limited to differential centrifugation, chromatography, electrophoresis and immunoprecipitation. In differential centrifugation complexes of molecules may be separated from uncomplexed molecules through a series of centrifugal steps due to the different sedimentation equilibria of complexes based on their different sizes and densities (see for example Rivas, G., and Minton, A.P., Trends Biochem. Sci. 18 (1993) 284-287). Standard chromatographic techniques may also be utilized to separate complexed molecules from uncomplexed ones. For example gel filtration chromatography separates molecules based on size and through the utilization of an appropriate gel filtration resin in a column format for example the relatively larger complex may be separated from the relatively smaller uncomplexed components.

Similarly the relatively different charge properties of the complex as compared to the uncomplexed molecules may be exploited to differentially separate the complex from the remaining individual reactants for example through the use of ion-exchange chromatography resins. Such resins and chromatographic techniques are well known to a person skilled in the art (see e.g. Heegaard, N.H., J. Mol. Recognit. 11 (1998) 141-148; Hage, D.S., and Tweed, S.A., J. Chromatogr. B. Biomed. Sci. Appl., 699

(1997) 499-525). Gel electrophoresis may also be employed to separate complexed molecules from unbound species (see e.g. Ausubel et al. (eds.), In: Current Protocols in Molecular Biology, J. Wiley & Sons, New York, 1999). In this technique protein or nucleic acid complexes are separated based on size or charge for example. In order to maintain the binding interaction during the electrophoretic process non-denaturing gels in the absence of reducing agent are typically preferred but conditions appropriate to the particular interactants will be well known to a person skilled in the art. Immunoprecipitation is another common technique utilized for the isolation of a protein-protein complex from solution (see e.g. Ausubel et al. (eds.), In: Current Protocols in Molecular Biology, J. Wiley & Sons, New York, 1999). In this technique all proteins binding to an antibody specific to one of the binding molecules are precipitated from solution by conjugating the antibody to a polymer bead that may be readily collected by centrifugation. The bound assay components are released from the beads (through a specific proteolysis event or other technique well-known in the art which will not disturb the protein-protein interaction in the complex) and a second immunoprecipitation step is performed, this time utilizing antibodies specific for the correspondingly different interacting assay component. In this manner only formed complexes should remain attached to the beads. Variations in no complex formation in both the presence and the absence of a test compound can be compared thus offering information about the ability of the compound to modulate interactions between the protein of the invention and its binding partner.

Also within the scope of the present invention are methods for direct detection of interactions between the protein of the invention and its natural binding partner and/or a test compound in a homogeneous or heterogeneous assay system without further sample manipulation. For example the technique of fluorescence energy transfer may be utilized (see e.g. U.S. Patent No. 5,631,169; U.S. Patent No. 4,868,103). Generally this technique involves the addition of a fluorophore label on a first 'donor' molecule (edge, test compound) such that its emitted fluorescent energy

will be absorbed by a fluorescent label on a second, 'acceptor' molecule (e.g. test compound), which in turn is able to fluoresce due to the absorbed energy.

Alternately the 'donor' protein molecule may simply utilize the natural fluorescent energy of tryptophan residues. Labels are chosen that emit different wavelengths of light such that the 'acceptor' molecule label may be differentiated from that of the 'donor'. Since the efficiency of energy transfer between the labels is related to the distance separating the molecules spatial relationships between the molecules can be assessed. In a situation in which binding occurs between the molecules, the fluorescent emission of the 'acceptor' molecule label in the assay should be maximal. An FET binding event can be conveniently measured through standard fluorometric detection means well-known in the art (e.g. using a fluorimeter). A test substance which either enhances or hinders participation of one of the species in the preformed complex will result in the generation of a signal variant to that of background. In this way test substances that modulate interactions between a protein of the invention and its binding partner can be identified in controlled assays.

In another embodiment modulators of nucleic acid molecule expression are identified in a method wherein a cell is contacted with a candidate compound and the expression of mRNA or protein encoded by a nucleic acid molecule is determined. The level of expression of mRNA or protein in the presence of the candidate compound is compared to the level of expression of mRNA or protein in the absence of the candidate compound. The candidate compound can then be identified as a modulator of nucleic acid molecule expression based on this comparison. For example when expression of nucleic acid molecule or protein is greater (statistically significantly greater) in the presence of the candidate compound than in its absence the candidate compound is identified as a stimulator of nucleic acid molecule expression. Conversely when expression of nucleic acid molecule mRNA or protein is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of nucleic acid

molecule expression. The level of nucleic acid molecule expression in the cells can be determined by methods described herein for detecting nucleic acid molecule mRNA or protein.

In another aspect the invention pertains to a combination of two or more of the assays described herein. For example a modulating agent can be identified using a cell- based or a cell free assay and the ability of the agent to modulate the activity of a protein of the invention can be further confirmed in viva, e.g., in a whole animal model for cellular transformation and/or tumorigenesis.

This invention further pertains to novel agents identified by the above-mentioned screening assays. Accordingly it is within the scope of this invention to further use an agent identified as described herein in an appropriate animal model. For example an agent identified as described herein (e.g. a gene or protein modulating agent, an antisense gene nucleic acid molecule, a protein specific antibody or a protein binding partner according to the invention) can be used in an animal model to determine the efficacy, toxicity or side effects of treatment with such an agent.

Alternatively an agent identified as described herein can be used in an animal model to determine the mechanism of action of such an agent. Furthermore this invention pertains to uses of novel agents identified by the above-described screening assays for treatments as described herein.

It is understood that appropriate doses of small molecule agents and protein or polypeptide agents depends upon a number of factors within the knowledge of the ordinarily skilled physician, veterinarian or researcher. The dose(s) of these agents will vary for example depending upon the identity, size and condition of the subject or sample being treated, further depending upon the route by which the composition is to be administered if applicable and the effect which the practitioner desires the agent to have upon the nucleic acid or polypeptide of the invention. Exemplary doses

of a small molecule include milligram or microgram amounts per kilogram of subject or sample weight (e.g. about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram or about 1 microgram per kilogram to about 50 micrograms per kilogram). Exemplary doses of a protein or polypeptide include gram, milligram or microgram amounts per kilogram of subject or sample weight (e.g. about 1 microgram per kilogram to about 5 grams per kilogram about 100 micrograms per kilogram to about 500 milligrams per kilogram or about 1 milligram per kilogram to about 50 milligrams per kilogram). It is furthermore understood that appropriate doses of one of these agents depend upon the potency of the agent with respect to the expression or activity to be modulated. Such appropriate doses can be determined using the assays described herein. When one or more of these agents is to be administered to an animal (e.g. a human) in order to modulate expression or activity of a polypeptide or nucleic acid of the invention a physician, veterinarian or researcher can for example prescribe a relatively low dose at first subsequently increasing the dose until an appropriate response is obtained. In addition it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific agent employed, the age, body weight, general health, gender and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral e.g. intravenous, intradermal, subcutaneous, oral (e.g. inhalation), transdermal (topical), transmucosal and rectal administration. Solutions or suspensions used for parenteral, intradermal or subcutaneous application can include the following components a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents, antibacterial agents such as benzyl alcohol or methyl parabens, antioxidants

such as ascorbic acid or sodium bisulfite, chelating agents such as ethylenediamine-tetraacetic acid, buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. For intravenous administration suitable carriers include physiological saline, bacteriostatic water, Cremophor EL (BASF; Parsippany, NJ) or phosphate buffered saline (PBS). In all cases the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing for example water ethanol, polyol (for example glycerol, propylene glycol and liquid polyethylene glycol and the like) and suitable mixtures thereof. The proper fluidity can for example be maintained by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents for example parabens, chlorobutanol, phenol, ascorbic acid, thimerosal and the like. In many cases it will be preferable to include isotonic agents for example sugars, polyalcohols such as mannitol, sorbitol or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption for example aluminium monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g. a polypeptide or antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above as required followed by filtered sterilization. Generally dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and then incorporating the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed.

Pharmaceutically compatible binding agents and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients or compounds of a similar nature. A binder such as microcrystalline cellulose, gum tragacanth or gelatin, an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel or corn starch, a lubricant such as magnesium stearate or Sterotes, a glidant such as colloidal silicon dioxide, a sweetening agent such as sucrose or saccharin or a flavouring agent such as peppermint, methyl salicylate or orange flavouring.

For administration by inhalation the compounds are delivered in the form of an aerosol spray from a pressurized container or dispenser which contains a suitable propellant, e.g. a gas such as carbon dioxide or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art and include for example for transmucosal administration, detergents, bile salts and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g. with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body such as a controlled release formulation including implants and microencapsulated delivery systems.

Biodegradable biocompatible polymers can be used such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art.

The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes having monoclonal antibodies incorporated therein or thereon) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to persons skilled in the art for example as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

For antibodies the preferred dosage is 0.1 mg/kg to 100 mg/kg of body weight (generally 10 mg/kg to 20 mg/kg). If the antibody is to act in the brain, a dosage of 50 mg/kg to 100 mg/kg is usually appropriate. Generally partially human antibodies and fully human antibodies have a longer half-life within the human body than other antibodies. Accordingly lower dosages and less frequent administration is often possible. Modifications such as lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration. A method for lipidation of antibodies is described by Cruikshank, W.W., et al., J. Acquired Immune Deficiency Syndromes and Human Retrovirology 14 (1997) 193-203.

VI. Pharmacogenomics

Agents or modulators which have a effect on the expression of the phosphorylated polypeptide of the invention can be administered to individuals to treat cancer in the patient. In conjunction with such treatment, the pharmacogenomics (i. e. the study of the relationship between an individual's genotype and that individual's response to a foreign compound or drug) of the individual may be considered. Differences in metabolism of therapeutics can lead to severe toxicity or therapeutic failure by altering the relation between dose and blood concentration of the pharmacologically

active drug. Thus the pharmacogenomics of the individual permits the selection of effective agents (e.g. drugs) for prophylactic or therapeutic treatments based on a consideration of the individual's genotype. Such pharmacogenomics can further be used to determine appropriate dosages and therapeutic regimens. Accordingly the level of expression of the polypeptide of the invention in an individual can be determined to thereby select appropriate agent(s) for therapeutic or prophylactic treatment of the individual.

Therefore, the invention considers s method of selecting a composition for inhibiting the progression of cancer in a patient, the method comprising:

a) providing a sample comprising cancer cells from the patient;
b) separately exposing aliquots of the sample in the presence of a plurality of test compounds;
c) comparing expression of the polypeptide according to the invention or a peptide fragment according to the invention in each of the aliquots; and
d) selecting one of the test compositions which alters the level of expression of the polypeptide in the aliquot containing that test composition, relative to other test compositions.

Particularly preferred in this context is also to determine the level of phosphorylated vs. unphosphorylated polypeptide according to the invention with and without test compound.

Pharmacogenomics deals with clinically significant variations in the response to drugs due to altered drug disposition and abnormal action in affected persons. See e.g. Linder, M.W., et al., Clin. Chem. 43 (1997) 254-266. In general two types of pharmacogenetic conditions can be differentiated. Genetic conditions transmitted as a single factor altering the way drugs act on the body are referred to as "altered drug action." Genetic conditions transmitted as single factors altering the way the body acts on drugs are referred to as "altered drug metabolism". These pharmacogenetic conditions can occur either as rare defects or as polymorphisms. For example

glucose-6-phosphate dehydrogenase (G6PD) deficiency is a common inherited enzymopathy in which the main clinical complication is haemolysis after ingestion of oxidant drugs (anti-malarials, sulfonamides, analgesics, nitrofurans) and consumption of Lava beans.

As an illustrative embodiment the activity of drug metabolizing enzymes is a major determinant of both the intensity and duration of drug action. The discovery of genetic polymorphisms of drug metabolizing enzymes (e.g. N-acetyltransferase 2 (NAT 2) and cytochrome P450 enzymes CYP2D6 and CYP2C19) has provided an explanation as to why some patients do not obtain the expected drug effects or show exaggerated drug response and serious toxicity after taking the standard and safe dose of a drug.

These polymorphisms are expressed in two phenotypes in the population the extensive metabolized (EM) and poor metabolizes (PM). The prevalence of PM is different among different populations. For example the gene coding for CYP2D6 is highly polymorphic and several mutations have been identified in PM which all lead to the absence of functional CYP2D6. Poor metabolizers of CYP2D6 and CYP2C19 quite frequently experience exaggerated drug response and side effects when they receive standard doses. If a metabolite is the active therapeutic moiety a PM will show no therapeutic response as demonstrated for the analgesic effect of codeine mediated by its CYP2D6 formed metabolite morphine. The other extreme are the so called ultra-rapid metabolizers who do not respond to standard doses. Recently the molecular basis of ultra-rapid metabolism has been identified to be due to CYP2D6 gene amplification.

Thus the level of expression of the polypeptide according to the invention in an individual can be determined to thereby select appropriate agent(s) for therapeutic treatment of the individual. In addition pharmacogenetic studies can be used to apply genotyping of polymorphic alleles encoding drug-metabolizing enzymes to the

identification of an individual's drug responsiveness phenotype. This knowledge when applied to dosing or drug selection can avoid adverse reactions or therapeutic failure and thus enhance therapeutic or prophylactic efficiency when treating a subject with a modulator of expression of the polypeptide of the invention.

VII. Monitoring Clinical Trials

Monitoring the influence of agents (e.g. drug compounds) on the level of expression of the polypeptide of the invention can be applied not only in basic drug screening, but also in clinical trials. In a preferred embodiment the present invention provides a method for monitoring the effectiveness of treatment of a subject with an agent (e.g. an agonist, antagonist, peptidomimetic, protein, peptide, nucleic acid, small molecule or other drug candidate) comprising the steps of (i) obtaining a pre-administration sample from a subject prior to administration of the agent, (ii) detecting the level of expression of the phosphorylated polypeptide according to the invention in the pre-administration sample, (iii) obtaining one or more post-administration samples from the subject, (iv) detecting the level of expression of the nucleic acid molecule in the post-administration samples, (v) comparing the level of expression of the polypeptide in the pre-administration sample with the level of expression of the polypeptide in the post-administration sample or samples and (vi) altering the administration of the agent to the subject accordingly. For example increased administration of the agent can be desirable to increase expression of the polypeptide to higher levels than detected i.e. to increase the effectiveness of the agent. Alternatively decreased administration of the agent can be desirable to decrease expression of the polypeptide according to the invention to lower levels than detected i. e. to decrease the effectiveness of the agent.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1: Sequence alignment between the sequences of p27, p21 and p57 (Program Gene Jockey II, Fixed and floating gap penalty: 10; PAM 250; Gap penalty: 5; > Window size: 5; Number of best diagonals: 3 ktupe:1.

Figure 4.1: Comparison of the inhibitory activity of $p27^{wt}$ and the CDK-binding-deficient mutant $p27^{\Delta CKI}$

In order to determine the inhibitory activity of both proteins a histone H1-kinase assay was carried out using active recombinant CDK2/cyclin A from insect cells. The two inhibitor proteins $p27^{wt}$ and $p27^{\Delta CKI}$ were expressed recombinantly in E. coli and purified as described under 4.1. The amount of both proteins was equalised by means of Coomassie staining after SDS-PAGE separation of the proteins. A titration was carried out with the same initial amounts of the two inhibitors. The activity of the inhibitor was determined by means of the incorporation of [α-^{32}P]ATP into the CDK2/cyclin A substrate histone H1. The inhibitory potential of p27 is characterized by the inactivation of CDK2/cyclinA-kinase and the associated reduction of the incorporation of phosphate into the histone H1. The titration was carried out with different amounts of p27 (1 µg, 0.5 µg, 10 ng and 0.5 ng) at constant amounts of kinase (50 ng) and substrate (2 µg). The radiogram of the dried gel of the kinase reactions separated by SDS-PAGE is shown. (A) Titration of $p27^{wt}$ (B) Titration of the CDK-binding-deficient mutant $p27^{\Delta CKI}$.

Figure 4.2: Real-time micrograph of a complete cell division of a HeLa cell transfected with the CDK binding-deficient mutant p27.
HeLa cells were transfected with the YFP fusion construct of the CDK binding-deficient mutant of p27, $p27^{\Delta CKI}$, and cell division was documented in serial photographs on a real-time microscope. The cells were photographed at intervals of 15 min in the fluorescence channel (excitation wavelength 498 nm) and in the phase

contrast channel. The pictures were subsequently compiled into a serial sequence. The CDK binding-deficient mutant of p27 exhibited no cell cycle arrest compared to the analogous YFP fusion construct of $p27^{wt}$ (data not shown). The dividing cell is labelled with an arrow.

Figure 4.3.: Identification of new binding partners of the Cip/Kip inhibitor proteins

The inhibitor proteins $p27^{wt}$, $p27\Delta^{CKI}$, p57 purified from *E. coli* were coupled covalently to an activated Affi-gel 10 matrix. The immobilized proteins were used as a bait for interacting proteins from a HeLa crude extract. Cell extract from 25 l dense HeLa suspension culture (1×10^6 cells/ml) was used per immobilized protein. The silver-stained gels of the interaction partners of $p27^{wt}$ of the CDK binding-deficient mutants $p27^{\Delta CKI}$, p21 and p57 separated by SDS-PAGE are shown. The postulated CDKs and cyclins were identified by immunoblot analysis (see figure 4.4). The other unknown proteins were determined from the $p27^{wt}$ lane by mass spectroscopy and peptide sequencing. 1: potential interactant of p57, ca. 16 kDa; 2: $p19^{SKP1}$; 3: potential interactant; 4: Grb2; 5: CDKs (CDK1, CDK2 and CDK3); 6: PCNA, specific interactant of p21; 7: heat shock protein; 8: β-actin, 9: Novel F-box protein; 10: cyclin E1; 11: cyclin A1; 12: cyclin B1; 13, 14 and 15 heat shock proteins.

Figure 4.4: Identification of the CDKs and cyclins obtained in the pull down with $p27^{wt}$.

The Coomassie stained protein bands (see also figure 4.3) obtained in the pull down were cut out with the polyacrylamide, boiled in 2 x SDS sample buffer and separated by SDS-PAGE. Cyclins, CDKs and $p19^{SKP1}$ were identified by immunoblot analysis using specific antibodies. Lane 1: HeLa protein extract.

Figure 4.7: Examination of the interaction of Grb2 with the Cip/Kip inhibitor family.

p27, p21 and p57 were expressed as hexahistidine fusion proteins in *E. coli*. Grb2

was produced recombinantly in *E. coli* without a fusion tag. Equal amounts of the bacterial crude extracts of the respective inhibitor protein and of Grb2 were incubated together. The histidine fusions of the inhibitors were precipitated by nickel-chelate Sepharose. The Grb2 associated with the inhibitors was detected by Coomassie staining after separating the proteins by SDS-PAGE. Free nickel-chelate Sepharose which was incubated with a comparable amount of Grb2 crude extract served as a control (K).

Figure 4.8: Analysis of the binding site of Grb2 in p27.
(A) $p27^{wt}$ (lane 1) and two shortened forms of p27, the N-terminal half of p27, $p27^{N\text{-}t}$ (Aa 1-96) and the C-terminal half of p27, $p27^{C\text{-}t}$ (Aa 97-198, lanes 3 and 5) and the analogous shortening of CDK binding-deficient form of $p27^{CKI}$ (lane 2), $p27^{CKI\ N\text{-}t}$ (lane 4) were expressed as hexahistidine fusions in *E. coli*. Grb2 was produced recombinantly in *E. coli* without a fusion tag. Equal amounts of bacterial crude extracts of the respective inhibitor protein and of Grb2 were incubated together. The histidine fusions of the inhibitors were precipitated by nickel-chelate Sepharose. The Grb2 bound to the inhibitors was detected by Coomassie staining after separating the proteins by SDS-PAGE. Free nickel-chelate Sepharose which was incubated with a comparable amount of Grb2 crude extract (lane 6) served as a control.

(B) $p27^{wt}$ (lane 1) and a form of p27 in which the amino acids P94P95K96 required for the interaction with Grb2 were converted into G94G95A96 ($p27^{\Delta SH3}$, lane 2) were produced in *E. coli* as hexahistidine fusions. The interaction experiment with Grb2 crude extract was carried out as described under (A). Free nickel-chelate Sepharose also served as a control in this case (lane 3).

Figure 4.9: Characterization of the binding of the C- and N-terminal SH3 domains and the central SH2 domain of Grb2 to p27.
The isolated C- and N-terminal SH3 domain (lane 1 and 2) and the isolated central SH2 domain (lane 3) of Grb2 were expressed in *E. coli* as GST fusion proteins and

specifically concentrated by means of glutathione Sepharose. These domains immobilized on glutathione Sepharose were incubated with recombinant purified p27 in an equal quantity ratio. The immobilized domains were washed, separated by SDS-PAGE and blotted (top, amido black staining). The interaction was analysed immunologically using an antibody directed against p27 (bottom). GST immobilized on glutathione Sepharose which was incubated with a comparable amount of p27 served as a control (lane 4).

Figure 4.10: Grb2 is displaced from its binding to p27 by CDK2/cyclin A.
The CDK2/cyclin A complex (lane 1) was produced in Sf9 insect cells by overexpressing the individual proteins using recombinant baculoviruses and purified. The Grb2-p27 complex (lane 2) was expressed in bacteria and purified therefrom. Both complexes were incubated together. The resulting complexes were separated according to their Stoke's radius by gel filtration on a Superdex 200pg 26/60 column. The column elution profile of the isocratic run (bottom) shows three peaks of the amount of protein measured at 280 nm. The fractions (red) of the column eluate were separated by SDS-PAGE and stained with Coomassie (bottom).

Figure 4.11: Comparison of the inhibitory activity of Grb2-bound and free p27.
The amounts of purified, free p27 and the purified complex p27-Grb2 (at a stoichiometry of 1:1) were adjusted after SDS-PAGE separation and Coomassie staining. The same amounts of free and complex-bound p27 were compared in order to determine their inhibitory activity in a kinase assay using CDK2/cyclin A. The activity of the inhibitor is determined by means of the incorporation of [γ-^{32}ATP] into the CDK2/cyclin A substrate histone H1. The inhibitory activity of p27 is characterized by the inactivation of the CDK2/cyclin A kinase and the associated reduction in the incorporation of phosphate into histone H1. The titration was carried out with different amounts of inhibitor (500 ng, 100 ng, 25 ng, 1 ng) at constant amounts of kinase (50 ng) and substrate (2 µg). A sample without inhibitor or

without the complexed inhibitor (-) served as an activity comparison. The radiogram of the dried gel of the kinase reaction separated by SDS-PAGE is shown.

Figure 4.12: Figure of the location of Grb2 in HeLa cells and its location in HeLa cells cotransfected with p27 and Grb2.
Grb2 was cloned as an N-terminal fusion with GFP in order to examine the location of Grb2. 24 h after transfection of adherent HeLa cells the location of Grb2 was analysed microscopically in fluorescent light and phase contrast. The GFP empty vector served as a control. In order to examine whether p27 can change the location of Grb2, GFP-Grb2 and p27-HA were cotransfected and also examined microscopically 24 h after transfection. A HA fusion construct of p21 and the HA fusion construct of the SH3-domain-binding-deficient mutant $p27^{\Delta SH3}$ served as controls. In order to check the transfection efficiency, p27-HA was detected in the rhodamine fluorescence channel by means of the specific HA antibody.

Figure 4.13: Systematic interaction analysis of isolated SH3 domains of various proteins with p27.
The isolated SH3 domains were expressed in *E. coli* as GST fusion proteins and concentrated by glutathione Sepharose. The amounts of protein and the isolated domains were adjusted by of Coomassie staining after SDS-PAGE separation. Identical amounts of SH3 domains and recombinant purified p27 immobilized on glutathione Sepharose were incubated together. Interactions were analysed by separating the reaction mixtures by SDS-PAGE and detection in an immunoblot using an antibody directed against p27. The isolated C- and N-terminal SH3 domain of Grb2 (lanes 21 and 22 (A) and 18 and 19 (B)) and the free GST immobilized on glutathione Sepharose (lanes 23 (A), 20 (B)) served as controls for the specificity of the interaction.

(A) Analysis of the isolated SH3 domains of different kinases of signal transduction: 1: Abl, 2: Src, 3: Yes, 4: Fyn, 5: Lyn, 6: Hck, 7: Lck, 8: Csk, 9: Itk, 10: p85, 11:

CIP4, 12: MAGUK2, 13: CD2BP1, 14: GapC1, 15: FBP17, 16: Fe65WW, 17: Flaf1WW, 18: Flaf2-1, 19: Flaf2-3, 20: Flaf3, 21: Grb2 (N-t SH3), 22: Grb2(C-t SH3), 23: GST control.

Figure 4.14: Characterization of the interaction between p27 and Lyn *in vivo*. Adherent HeLa cells were transfected with various HA fusion constructs of p27, $p27^{wt}$-HA (lane 1, 4 and 6), $p27^{Y1Y2Y3/F}$-HA (lane 2, 7 and 8) and $p27^{\Delta SH3}$-HA (lane 3). They were additionally cotransfected with the longer isoform (p56) of the tyrosine kinase Lyn in lanes 1, 2, 3, 4, 5 and 8. The cells were lysed in IP buffer 24 h after transfection and the amount of protein in the extracts was matched. p27 was precipitated by specific antibodies (Sc C-19). Interaction with Lyn was analysed in an immunoblot using an antibody directed against Lyn. Transfection with an SH3 domain-binding-deficient mutant of p27, $p27^{\Delta SH3}$ (fig. 4.14, lane 3) and an immunoprecipitation with unspecific immunoglobulins from normal rabbit serum (fig. 4.14, lane 4) served as controls for the specificity of the interaction. This immunoprecipitation was also carried out with $p27^{wt}$-HA and Lyn cotransfected HeLa cell extract. Additional immunoprecipitations were carried out with endogenous p27 and overexpressed Lyn (fig. 4.14, lane 5) as well as with the purified plasma membrane of the transfection with $p27^{Y1Y2Y3/F}$-HA and Lyn (fig. 4.14, lane 8). The plasma membranes were solubilized in RIPA buffer and precipitated with a p27-specific antibody after a ten-fold dilution with IP buffer.

Figure 4.15: Determination of the in vivo phosphorylation state of p27 by 2-D gel electrophoresis.

(A) Isolation of endogenous p27 from MCF7 cells: MCF7 cells from ten confluent 15 cm plates were taken up in IP buffer and lysed by ultrasound. The crude extract was boiled for 10 min at 98°C, cooled for 10 min on ice and centrifuged. The remaining heat-stable proteins were precipitated from the supernatant with acetone. This precipitate was dissolved in PBS and the proteins were separated according to their Stoke's radius by gel filtration. The fractions containing p27 were determined

immunologically and the corresponding fractions were pooled (fractions 9-11). The individual fractions of the protein fractionation (top) and the specific antibody detection (bottom) are shown, lane 1: applied boiled crude extract.

(B) Separation of p27 in the 2-D gel: The combined fractions from the gel filtration were again precipitated with acetone and taken up in the buffer for the isoelectric focussing. The focussing was carried out using a non-linear, immobilized gel strip (pH 3-10) at 100000 V/h. The focussed proteins were subsequently separated in the second dimension by SDS-PAGE. The phosphorylation state of p27 was analysed in the immunoblot using p27-specific and phospho-specific antibodies.

Figure 4.16: Analysis of *in vitro* on tyrosine-phosphorylated p27 by thin layer chromatographic separation of the phosphoamino acids of hydrolysed p27.
(A) Recombinant p27 was phosphorylated with [γ-^{32}P]ATP *in vitro* using the recombinantly produced Abl tyrosine kinase fragment Abl^{KD+SH3}. The radioactively labelled p27 was incubated for 30 min at 30°C in MCF7 crude extract in the presence of phosphatase inhibitors and subsequently precipitated with a specific p27 antibody. The sample was separated by SDS-PAGE, blotted on a PVDF membrane and exposed to an X-ray film (lane 1). The membrane was superimposed on the X-ray film and the phosphorylated p27 was cut out of the membrane. The remaining membrane was exposed again in order to check the correct position of the excised p27 (lane 2).

(B) Two-dimensional separation of the phosphoamino acids of p27: The membrane fragment was rehydrated in methanol, washed and treated for 1 h with 6 N HCl at 110°C. The resulting protein hydrolysate was removed and dried under a vacuum, dissolved in the mobile buffer of the first dimension and applied to a cellulose thin layer plate together with a phosphoamino acid standard. The phosphoamino acids were separated electrophorectically in two dimensions. The first dimension was carried out for 35 min at pH 1.9 and 1.5 kV. The plate was dried and the amino acids

were separated for 30 min in the second dimension at pH 3.5 and 1.3 kV. The phosphoamino acid standard was subsequently made visible by ninhydrin staining. The phosphorylated amino acids were detected by exposing the cellulose plate to an X-ray film.

Figure 4.18: Metabolic labelling of adherent HeLa cells with [^{32}P]-ortho-phosphate and thin layer chromatographic analysis of the phosphoamino acids of overexpressed and stabilized p21 and p27.
(A) A 250 ml bottle of adherent HeLa cells was transfected with p21 and p27 and 24 h after transfection it was metabolically labelled for 4 h with 5 mCi (0.5 mCi/ml) [^{32}P]-*ortho*-phosphate. The cells were additionally cultured in the presence of MG132, LLnL, ocadeic acid and Na *ortho*-vanadate to inhibit the proteasome and cell phosphatases. During the last 30 min of the metabolic labelling the cells were also cultured with Na *meta*-vanadate. p21 and p27 were immunoprecipitated by specific antibodies. The mixtures were separated on SDS-PAGE and blotted on a PVDF membrane. The membrane was exposed to an X-ray film (lane 1a and 2a) and the membrane was superimposed on radioactive signals of the film. p21 and p27 were cut out of the membrane. The remaining membrane was again exposed to verify the excised proteins (lane 1b and 2b).

(B) Two-dimensional separation of the phospamino acids of overexpressed p21 and p27: The membrane fragments were rehydrated in methanol, washed and treated for 1 h with 6 N HCl at 110°C. The resulting protein hydrolysate was removed and dried under a vacuum, dissolved in the mobile buffer of the first dimension and applied to a cellulose thin layer plate together with a phosphoamino acid standard. The phosphoamino acids were separated electrophorectically in two dimensions. The first dimension was carried out for 35 min at pH 1.9 and 1.5 kV. The plate was dried and the amino acids were separated for 30 min in the second dimension at pH 3.5 and 1.3 kV. The phosphoamino acid standard was subsequently made visible by

ninhydrin staining. The phosphorylated amino acids were detected by exposing the cellulose plate to an X-ray film.

Figure 4.19: Comparison of phosphorylation efficiency of the tyrosine kinases AbI and Src for the substrates p27 and Sam68.
p27 produced and purified from *E. coli* and the commercial tyrosine kinase substrate Sam68 were used as substrates in a tyrosine kinase assay. The experiment compared the ability of the shortened tyrosine kinase Abl^{KD+SH3} produced in bacteria and of the complete tyrosine kinase Src (Src^{rec}) that was also produced in and purified from bacteria, to phosphorylate p27. In order to compare the activity of the tyrosine kinase Src^{rec}, Src (Src^{sf9}) produced recombinantly in Sf9 cells was used to phosphorylate the substrates p27 and Sam68. The kinase treatments were carried out for 2 h at 30°C in kinasation buffer II. The Coomassie-stained SDS-PAGE separated gel of the kinase reactions (top) and the radiogram of the dried gel (bottom) are shown.

Figure 4.20: Comparison of the ability to phosphorylate the tyrosines of the Cip/Kip inhibitor proteins *in vitro*.
p21, p27 and p57 that were produced in *E. coli* and purified, were phosphorylated with the recombinant, shortened tyrosine kinase Abl^{KD+SH3}. The kinase reaction was carried out for 2 h at 30°C. The effectiveness of the phosphorylation of the various inhibitor proteins was estimated by incorporating [γ-^{32}P]ATP into the respective protein. The nucleotide exchange factor RanGAP1 and the BSA which was added to the kinase reactions for stabilization served as controls of the specificity of phosphorylation. The Coomassie-stained gel of the kinase reactions (top) and the radiogram of the dried gel (bottom) are shown.

Figure 4.21: Determination of the tyrosines of p27 that can be phosphorylated *in vitro* by Abl^{KD+SH3}.
In order to characterize the tyrosines in p27 that can be phosphorylated, the three tyrosines of p27 were constitutively replaced by phenylalanine in various

combinations. The various substitution mutants of p27 and $p27^{wt}$ were produced recombinantly in *E. coli* and purified. The same amounts of these proteins were treated with kinase for 2 h at 30°C using the recombinant, shortened tyrosine kinase Abl^{KD+SH3}. The effectiveness of the phosphorylation of the various substrate proteins was determined by measuring the incorporation of [γ.^{32}P]ATP into the respective protein. A form of p27 in which all tyrosines were converted into phenylalanine ($p27^{Y1Y2Y3/F}$) was used as a control for the absence of a serine/ threonine kinase activity of the substrates. The Coomassie-stained gel of the kinase reactions (top) and the radiogram of the dried gel (bottom) are shown.

(A) Determination of the ability to phosphorylate tyrosine at position 74 and the two neighbouring tyrosines at positions 88 and 89. In this experiment $p27^{wt}$ was compared with the substitution mutants $p27^{Y1/F}$ and $p27^{Y2Y3/F}$.

(B) Determination of the ability to simultaneously phosphorylate tyrosines 88 and 89. In this case $p27^{wt}$ was compared with the substitution mutants $p27^{Y1Y2/F}$ and $p27^{Y1Y3/F}$.

Figure 4.22: Characterization of the binding of tyrosine-phosphorylated p27 to the CDK2/cyclin A complex.

(A) The CDK2/cyclin A complex that was prepared by overexpressing the individual proteins using recombinant baculoviruses in SF9 insect cells and purified, was admixed and incubated with an excess of tyrosine-phosphorylated and unphosphorylated p27 (at a stoichiometry of 1:1 for both inhibitors). In addition $p27^{wt}$ was phosphorylated *in vitro* with the shortened, recombinant tyrosine kinase Abl^{KD+SH3}. The tyrosine-phosphorylated p27 was boiled and centrifuged in order to separate the kinase. The sample was subsequently diafiltered in order to separate excess ATP. The trimeric complex CDK2/cyclin A-p27 was separated according to the Stoke's radius by gel filtration using a Superdex 200pg 26/60 column in order to determine whether tyrosine-phosphorylated p27 can bind to CDK2/cyclin A. The

fractions of the column eluate were separated by SDS-PAGE and stained with Coomassie in order to analyse the composition of the complex (top). In order to determine the phospho state of complexed and free p27, the fractions from the gel filtration were analysed by immunoblot using a specific antibody directed against phosphotyrosine (bottom).

(B) The specificity of the phosphoantibody that was used was tested with a p27 phosphorylated *in vitro* by Src (recombinant from bacteria) and with a control-treated p27.

Figure 4.23: Determination of the inhibitory activity of *in vitro* tyrosine-phosphorylated p27 compared to unphosphorylated p27 and to $p27^{Y123/F}$. $p27^{wt}$ and $p27^{Y123/F}$ were expressed recombinantly in *E. coli* and purified. A portion of the purified $p27^{wt}$ was phosphorylated *in vitro* with the shortened, recombinant tyrosine kinase Abl^{KD+SH3}. The tyrosine-phosphorylated p27 was boiled and centrifuged in order to separate the kinase. The sample was subsequently diafiltered in order to separate excess ATP from the kinasation reaction. The amounts of all three proteins was adjusted on the basis of Coomassie staining after SDS-PAGE. A histone H1 kinase assay using active human CDK2/cyclin A isolated from insect cells was carried out in order to characterized the inhibitory activity of the proteins. A titration was carried out with the same initial amounts of the inhibitors in order to determine the kinetics of inhibition. In this method the activity of the inhibitor was measured by means of the incorporation of [γ-^{32}P]ATP into the CDK2/cyclin A substrate histone H1. The inhibitory activity of p27 is characterized by the inactivation of CDK2/cyclin A kinase and the associated reduction in the incorporation of phosphate into histone H1. The titration was carried out using seven points in each case of decreasing amounts of the respective inhibitor at constant amounts of kinase (50 ng) and substrate (2 µg). A kinase reaction without inhibitor (-) served as a control of the activity. The Coomassie-stained gels of the kinase reactions (top) and the radiograms of dried gels (bottom) are shown.

(A) Titration kinetics for $p27^{wt}$ and tyrosine-phosphorylated p27.

(B) Titration kinetics for $p27^{Y123/F}$ and tyrosine-phosphorylated p27. The kinetics that were determined for the respective inhibitors are summarized graphically in (C). A kinase assay was carried out using pRb as a substrate to confirm the kinetics for tyrosine-phosphorylated p27 and for another substrate of CDK2/cyclin A. The inhibitory potential of $p27^{wt}$ and of *in vitro* tyrosine-phosphorylated p27 was determined under identical conditions to (A) and (B) in a kinase reaction using active CDK2/cyclin A and pRb (D).

Figure 4.24: Characterization of increased serine/threonine phosphorylation of tyrosine-phosphorylated p27 by CDK2/cyclin A.

$p27^{wt}$ was phosphorylated *in vitro* with Abl^{KD+SH3}. The tyrosine-phosphorylated p27 was boiled and centrifuged in order to separate the kinase. The sample was subsequently diafiltered in order to separate excess ATP. The same amounts of tyrosine-phosphorylated p27, $p27^{wt}$ and of $p27^{Y1237F}$ (top) were incubated for 30 min at 30°C with active CDK2/cyclin A. The ability of the CDK2/cyclin A complex to phosphorylate the inhibitor p27 was determined by the incorporation of [γ-^{32}P]ATP into p27. The incorporation of [γ-^{32}P]ATP into the CDK2/cyclin A substrate histone H1 (bottom) served as a control of the phosphorylation reaction.

Figure 4.25: Examination of the ability to phosphorylate the tyrosines of p27 bound to CDK2/cyclin A.

The recombinant, purified CDK2/cyclin A complex from insect cells and purified p27 bacteria were incubated together and the trimeric complex (at a stoichiometry of 1:1:1) was separated from unbound proteins by gel filtration. A portion of the purified trimeric complex was boiled for 10 min and centrifuged in order to separate CDK2/cyclin A. Free p27 and an identical amount of CDK2/cyclin A-bound p27 were phosphorylated *in vitro* with Abl^{KD+SH3} in order to determine the accessibility of bound p27 to the tyrosine kinase. The serine/threonine phosphorylation of p27

itself mediated by the CDK2/cyclin A complex was analysed in a kinasation reaction without tyrosine kinase (lane 2). This activity was subtracted from the measured total activity. The ability of the tyrosine kinase to phosphorylate the inhibitor p27 was characterized by incorporation of [γ-^{32}P]ATP into p27. The Coomassie-stained gel of the kinase reaction (top) and the radiogram of the dried gel (bottom) are shown.

Figure 4.26: Investigation of the mechanism of the dissociation of tyrosine kinase Lyn from its substrate p27 *in vitro*.
(A) The isolated SH3 domain of the tyrosine kinase Lyn was expressed in *E. coli* as a GST fusion protein and concentrated by glutathione Sepharose. The domain immobilized on glutathione Sepharose was incubated with recombinant, purified $p27^{wt}$ (lane 2), $p27^{Y123/F}$ (lane 3), $p27^{\Delta SH3}$ (lane 4) and with a tyrosine-phosphorylated p27 (lane 1). This p27 (lane 1) was treated with kinase *in vitro* using the shortened, recombinant tyrosine kinase Abl^{KD+SH3}, and boiled and centrifuged in order to separate the kinase. The sample was subsequently diafiltered in order to separate excess ATP. The interaction experiment was separated by SDS-PAGE and blotted (top). The interaction was analysed immunologically using a specific antibody directed against p27 (bottom). GST-immobilized on glutathione Sepharose which had been incubated with a comparable amount of tyrosine-phosphorylated p27 served as a control (lane 5).

(B) The amount of protein of the various forms of p27 produced recombinantly and of tyrosine-phosphorylated p27 were matched on the basis of Coomassie staining of the proteins separated by SDS-PAGE. The efficiency of the tyrosine-phosphorylation of p27 was detected by a slight increase in the size of phospho-p27 compared to unphosphorylated p27. Lane 1: tyrosine-phosphorylated p27, lane 2: $p27^{wt}$, lane 3: $p27^{Y123/F}$, lane 4: $p27^{\Delta SH3}$.

Figure 4.27: Investigation of the inactivation of p27 by tyrosine phosphorylation of the inhibitor *in vivo*.

A 10 cm dish of adherent HeLa cells was in each case cotransfected with HA fusion constructs of $p27^{wt}$-HA (lane 1) and $p27^{Y1Y2Y3/F}$-HA (lane 2) and additionally with the longer isoform (p56) of a constitutively active form of the tyrosine kinase Lyn. The cells were lysed in IP buffer 24 h after the transfection and the amounts of protein were equalised. p27-HA was specifically immunoprecipitated from the extracts with a specific HA antibody and cyclin A was specifically immunoprecipitated from parallel preparations. An immobilized histone H1 kinase assay was carried out with the immunoprecipitated kinase to determine the inhibitory activity of the two transfected inhibitors $p27^{wt}$ and $p27^{Y1Y2Y3/F}$. The activity of the inhibitor immobilized on the antibody and protein A Sepharose is determined by incorporation of [γ-^{32}P]ATP into the CDK2/cyclin A substrate histone H1. The inhibitory activity of p27 is characterized by the inactivation of CDK2/cyclin A kinase and the associated reduction of the incorporation of phosphate into histone H1. The radiogram of the PVDF membrane of the kinase reactions separated by SDS-PAGE is shown. The amounts of endogenous CDKs and overexpressed inhibitors used in the kinase assay were determined with specific antibodies in the immunoblot analysis of the PVDF membrane of the kinase reactions.

Figure 4.28: Determination of the half-life of $p27^{Y123/F}$ and $p27^{\Delta SH3}$ compared to $p27^{wt}$.

(A) A 15 cm dish of adherent HeLa cells was transfected in each case with $p27^{wt}$, $p27^{\Delta SH3}$ and $p27^{Y123/F}$ and each of the transfected dishes was divided into equal portions between 4 cell culture flasks 5 h after the transfection. 16 h after dividing the cells, the cells were metabolically labelled for 1 h with 0.05 mCi/ml [^{35}S]-methionine and [^{35}S]-cysteine. The cells were cultured for a further 0, 1, 2 and 5 h in a medium containing non-radioactive methionine and cysteine and harvested. The cells were lysed and the amount of protein in the lysates were equalised. The crude extract was boiled for 10 min, cooled on ice and centrifuged. The radioactively labelled p27-HA fusions were immunoprecipitated by an antibody directed against

HA. The Coomassie staining of the separated precipitates (top) and the radiogram of the dried gel (bottom) is shown.

(B) The boiled crude extract from the depletion with HA-specific antibody was separated by SDS-PAGE and stained with Coomassie in order to determine the amounts of protein used and the efficiency of incorporation of [^{35}S]-methionine and [^{35}S]-cysteine (top). The incorporation was estimated by exposing the dried gel to an X-ray film (bottom).

Figure 4.29: Comparison of the inhibitory activity of p27^{wt}, p27$^{\Delta SH3}$ and various tyrosine substitution mutants of p27.
A histone H1 kinase assay with active, recombinant CDK2/cyclin A from insect cells was carried out to determine the inhibitory activity of the proteins. The inhibitory proteins p27^{wt}, p27$^{\Delta SH3}$, p27$^{Y1/F}$, p27$^{Y2Y3/F}$ and p27$^{Y1Y2Y3/F}$ were expressed recombinantly in *E. coli* and purified. The amounts of the proteins was matched on the basis of Coomassie staining after SDS-PAGE separation. A titration was carried out with the same initial amounts. The activity of the tested inhibitors is determined by means of the incorporation of [γ-^{32}P]ATP into the CDK2/cyclin A substrate histone H1. The inhibitory activity of p27 is characterized by the inactivation of CDK2/cyclin A kinase and the associated reduction of the incorporation of phosphate into histone H1. The titration was carried out in each case with two different amounts of the respective inhibitor (40 ng and 4 ng) at constant amounts of kinase and substrate. A reaction without inhibitor (-) served as a control of the activity of the kinase. The radiogram of the dried gel of the kinase reactions separated by SDS-PAGE is shown.

Figure 4.30: Investigation of the cellular location of p27^{wt}, p27$^{\Delta SH3}$ and p27$^{Y123/F}$
HA fusion constructs of p27$^{\Delta SH3}$, p27$^{Y123/F}$ and p27^{wt} were transfected in adherent HeLa cells. 24 h after transfection, the cells were stained immunocytologically with a HA-specific antibody and the location of overexpressed p27 was analysed in a

fluorescence microscope. The phase contrast photograph (top), the fluorescence channel (middle) and a superimposition of these two channels is shown for the respective inhibitor (bottom).

Figure 4.31: Characterisation of a rabbit polyclonal antibody spcific for p27 phosphorylated on residue tyrosine 88. HA-tagged p27, was transfected into human tissue culture cells in the presence or absence of Lyn or Bcr-Abl kinases. p27 of transfected cells was immunopreciupitated with a monoclonal HA antibody. Identical fractions of the IP were incubated with calf intestinal alkaline phosphatase (CIAP, left) or mock treated. Proteins were separated by SDS-PAGE and analysed using a polyclonal antibody raised against the phosphopeptide NH_3-CLPEF(Y-PO_4)YRPPR-COO^-, where (Y- PO_4) is phosphotyrosine (αPY88, top) and subsequently probed for p27 abundance using an antibody directed against a C-terminal peptide (α□27; bottom). The strong signal of the phosphospecific antibody ist lost after phosphatase treatment, confirming specificity of the signal obtained with the phosphospecific antibody.

Figure 4.32: p27 is phosphorylated on tyrosine residue 88 *in vivo*. HA-tagged p27, mutant HA-tagged p27 (where tyrosines 88 and 89 were exchanged to phenylalanine) were transfected into human tissue culture cells in the presence or absence of Lyn or Bcr-Abl. p27 of transfected cells was recovered by immunoprecipitation with a monoclonal HA antibody, immunoprecipitates were separated by SDS-PAGE and analysed using a polyclonal antibody raised against the phosphopeptide NH_3-CLPEF(Y-PO_4)YRPPR-COO^-, where (Y- PO_4) is phosphotyrosine (αPY88; top) and subsequently for p27 abundance using an antibody directed against a C-terminal peptide (C20; obtained from Santa Cruz, bottom).

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Example 1

1 and 2 General

If not stated otherwise the described methods were derived from Sambrook *et al.* (1989), *Current Protocols in Molecular Biology, Current Protocols in Protein Science* and *Current Protocols in Cell Biology.*

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals and consumables

If not stated otherwise chemicals and fine chemicals were obtained in analytical quality from Merck (Darmstadt), Sigma-Aldrich (Taufkirchen), SERVA (Heidelberg) and Roth (Karlsruhe). The water used in this study was prepared on a "Purelab Plus UV/UF" filter system from the USF Company (Ransbach – Baumbach). It is referred to in the following as ddH_2O.

3.1.1.1 Radiochemicals

Radiochemicals were obtained from Amersham Pharmacia Biotech (Freiburg).

3.1.1.2 Materials for filtration and dialysis

Centricon 5, 10 and 30	Amicon, Witten
Diaflow ultrafiltration membranes	Amicon, Witten
MF-Millipore membrane filter	Sigma-Aldrich, Taufkirchen
Spectra-Por, dialysis tubes	The Spectrum Companies, Gardena, USA
UH 100/10 and 30 ultra cartridges	Schleicher & Schüll, Dassel
Whatman 3 MM, filter paper	Maidstone, UK
Whatman GF/C, glass fibre filter	Maidstone, UK
centrifuge filter units 5K, 10K and 30K	Sigma-Aldrich, Taufkirchen

3.1.1.3 Chromatography matrices and ready-packed chromatography columns

Affi-Gel10	Bio-Rad Laboratories GmbH, Munich
CNBr-Sepharose 4B	Sigma-Aldrich, Taufkirchen
DC plates 20 X 20 cm, Cellulose	Merck, Darmstadt
Glutathione Sepharose 4B	Amersham Pharmacia Biotech, Freiburg
High Trap Q 1 ml and 5 ml	Amersham Pharmacia Biotech, Freiburg
High Trap SP 1 ml and 5 ml	Amersham Pharmacia Biotech, Freiburg
MonoQ HR 5/5	Amersham Pharmacia Biotech, Freiburg
ProBond Nickel-Chelating Resin	Invitrogen, Groningen, Netherlands
protein A Sepharose CL-4B	Sigma-Aldrich, Taufkirchen
protein G Sepharose 4 fast flow	Amersham Pharmacia Biotech, Freiburg
Sepharose-SP fast flow	Sigma-Aldrich, Taufkirchen
Sepharose-Q fast flow	Sigma-Aldrich,
Taufkirchen	
Sephasil C4µm ST 4.6/250 protein C4	Amersham Pharmacia Biotech, Freiburg
Superdex 200 HR 10/30	Amersham Pharmacia Biotech, Freiburg
Superdex 200 pg HiLoad 16/30	Amersham Pharmacia Biotech, Freiburg
Superdex 200 pg HiLoad 26/60	Amersham Pharmacia Biotech, Freiburg

3.1.1.4 Molecular weight and length standards

BenckMark Protein Ladder, protein size standard	Life Technologies, Karlsruhe
GeneRuler 100bp Ladder Plus, DNA size standard	MBI Fermentas, St. Leon-Rot
GeneRuler 1kb Ladder Plus, DNA size standard	MBI Fermentas, St. Leon-Rot
IEF Markers 3-10, SERVA liquid mix	Invitrogen, Groningen, Netherlands
MW-FG-200, gel filtration marker 12-200 kDa,	Sigma-Aldrich, Taufkirchen

3.1.1.5 Complex reagents and reaction sets (kits)

Big Dye Terminator Cycle Sequencing RR-Mix Applied Biosystems, Foster City, USA

Complete without EDTA, protease inhibitor mix, Roche

Mannheim

Deoxynucleotide set Sigma-Aldrich,

Taufkirchen

Effectene, Transfection reagent QIAGEN,

Hilden

Freund's Adjuvant, incomplete Sigma-Aldrich,

Taufkirchen

Glow, mounting medium Energene,

Regensburg

IPG buffer, pH 3-10 and pH 6-11 Amersham Pharmacia Biotech,

Freiburg

Micro BCA Protein Assay Reagent Kit Pierce, Rockford,

USA

pGEM-T Easy Vector System I and II Promega,

Mannheim

QIAGEN Plasmid Kit (Mini, Midi) QIAGEN,

Hilden

Rotiphorese Gel 30 Roth,

Karlsruhe

Super Signal West Femto Maximum Sensitivity Substrate Pierce, Rockford,

USA

TA Cloning Kit Invitrogen, Groningen,

Netherlands

TiterMax Gold, Adjuvant CytRxCorporation, Norcross,

USA

TNT-SP6/T7 Coupled Reticulocyte Lysate System Promega,

Mannheim

TOPO TA Cloning Kit Invitrogen, Groningen,

Netherlands

3.1.1.6 Consumables

3.1.2 Buffers and solutions

Amido black staining solution	0.2 % (w/v) amido black 10B, 10 % (v/v) methanol, 2 % (v/v) acetic acid
block solution	2 % (w/v) BSA in PBS
complete without EDTA, 50 x	1 tablet complete without EDTA – protease inhibitor mix (Roche) in 1 ml ddH_2O
Coomassie staining solution (SERVA)	50 % (v/v) methanol, 10 % (v/v) acetic acid, 0.1 % (w/v) SERVA Blue R
TLC buffer I	50 ml (88 %) formic acid, 156 ml glacial acetic acid make up to 2000 ml with ddH_2O, do not adjust pH which should be about 1.8 – 1.9
TLC buffer II	100 ml glacial acetic acid, 10 ml pyridine, 0.5 ml EDTA make up to 2000 ml with ddH_2O, adjust the pH to 3.5 with pyridine
DNA application buffer, 6 x	10 mM Tris/HCl, 50 mM EDTA, 1 % SDS (w/v), 30 % (w/v) glycerol, 0.1 % (w/v) bromophenol blue, pH 8.0
IP buffer	200 mM NaCl, 50 mM Tris, pH 7.5, 0.25 % (w/v) NP-40, 5 mM EDTA and 1 mM PMSF, 10 µg/ml Aprotinin, leupeptin, pepstatin A
Kinasation buffer I, 10 x	200 mM Tris/HCl, 75 mM $MgCl_2$, pH 7.2
Kinasation buffer II,	20 mM HEPES pH 7.5, 5 mM MgCl2, 1 mM $MnCl_2$, 0.05 % (w/v) NP-40, 7.15 mM β-mercaptoethanol, 125 µM Na *ortho*-vanadate, 10 µM PMSF, 0.1 µg/ml

	A, P, LP
NET gelatin block buffer, 10 x	1.5 M NaCl, 0.05 M EDTA, 0.5 M Tris pH 7.5, 0.5 % (w/v) Triton-X-100, 26 g/l gelatin, adjust to pH 7.5
P1	50 mM Tris/HCl, 10 mM EDTA, 100 µg/ml RNase A, pH 8.0
P2	200 mM NaOH, 1 % (w/v) SDS
P3	3 M K acetate, pH 5.5
paraformaldehyde solution	6 % (w/v) paraformaldehyde adjusted to pH 7.4 with 10 N NaOH; stored in the dark at 4°C; working solution 3 % paraformaldehyde in PBS
PBS	140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO4, adjust pH to 7.5 with NaOH
PBS-T	0.05 % (w/v) Tween-20 in PBS
PI staining solution	1 µg/ml propidium iodide, 0.1 % (v/v) Triton-X-100, 0.2 mg/ml RNase A in PBS
RIPA buffer	50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1 % (v/v) Nonidet P 40, 0.25 % (w/v) sodium deoxycholate, 0.1 % (w/v) SDS, pH 7.5
SDS electrophoresis buffer	25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS
SDS sample buffer, 10 x	209 mM Tris/HCl, 41 % (w/v) glycerol, 7.7 % (w/v) SDS, 0.003 % (w/v)

bromo-	phenol blue, 17 % (v/v) β-mercaptoethanol, pH 6.8
SDS sample buffer, 2 x	125 mM Tris/HCl, 17 % (w/v) glycerol, 4.1 % (w/v) SDS, 0.001 % (w/v) bromo- phenol blue, 2 % (v/v) β-mercaptoethanol, pH 6.8
SDS collecting gel buffer, 4 x	0.5 M Tris/HCl, 0.4 % (w/v) SDS, pH 6.8
SDS separating gel buffer, 4 x	1.5 M Tris/HCl, 0.4 % (w/v) SDS, pH 8.8
TBE	90 mM Tris, 90 mM boric acid, 2 mM EDTA
TBS	20 mM Tris/HCl, 150 mM NaCl, pH 7.5
TE	10 mM Tris/HCl, 1 mM EDTA, pH 8.0
Triton-X-100 solution	0.2 % (w/v) Triton-X-100 in PBS
transfer buffer	47.9 mM Tris, 38.6 mM glycine, 0.037 % (w/v) SDS, 20 % (v/v) methanol
TSS	10 % (w/v) polyethylene glycol 6000, 5 % (v/v) DMSO, 50 mM $MgCl_2$ in LB medium

3.1.2 Media and media additives for the tissue culture

3.1.3.1 Media components and media additives

5-bromo-2'-deoxyuridine	Sigma-Aldrich, Taufkirchen

stock solution 1 % (w/v) in PBS
L-cysteine Sigma-Aldrich, Taufkirchen
stock solution 25 mg/ml in PBS
Dulbeccos's Modified Eagle's Medium (DMEM) containing 4500 mg/l glucose, without sodium pyruvate Life Technologies, Karlsruhe
DMEM containing 4500 mg/ml glucose, without L-glutamine, sodium pyruvate, L-cysteine, L-methionine Life Technologies, Karlsruhe
foetal calf serum (FCS) and newborn calf serum (NCS) Sigma-Aldrich, Taufkirchen
L-glutamine (100 x) Life Technologies, Karlsruhe
Joklik's Medium Life Technologies, Karlsruhe
Lovastatin Merck, Darmstadt
stock solution 40 mM in ethanol, activated
MEM-Minimum Essential Medium Life Technologies, Karlsruhe
MEM-Non-essential amino acid solution Life Technologies, Karlsruhe
L-methionine Sigma-Aldrich, Taufkirchen
Minimum Essential Medium Eagle, without Na-phosphate Sigma-Aldrich, Taufkirchen
stock solution 25 mg/ml in PBS
sodium pyruvate (100 x) Life Technologies, Karlsruhe
Nocadazol Sigma-Aldrich, Taufkirchen

stock solution 5 mg/ml in DMSO	
penicillin/streptomycin (100 x)	Life Technologies, Karlsruhe
Sf-900 II SFM Basal Powdered Medium	Life Technologies, Karlsruhe
Sf-900 II Supplement	Life Technologies, Karlsruhe
thymidine	Sigma-Aldrich, Taufkirchen
stock solution 200 mM in PBS	
trypsin/EDTA (10 x)	Life Technologies, Karlsruhe

3.1.3.2 Media composition

standard medium	
for adherent HeLa, 293T and MCF7	DMEM containing 4500 mg/l glucose, 10 % (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin
for HS68	DMEM containing 4500 mg/l glucose, 10 % (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate
HeLa suspension cells	Joklik's medium, 5 % (v/v) NCS, 100 U/ml penicillin, 100 µg/ml streptomycin
Chase medium	standard medium containing 0.1 mg/ml L-methionine, 0.12 mg/ml L-cysteine, 25 mM HEPES/KOH, pH 7.4
Hunger medium	DMEM containing 4500 mg/l glucose, 10 % (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES/KOH, pH 7.4, without sodium pyruvate, L-cysteine, L-methionine

Phospholabel medium	Minimum essential Eagle's medium containing Earls' salts, L-glutamine, $NaHCO_3$ and containing 4.5 g/l glucose, 2 % (v/v) FCS without sodium phosphate
Puls-Medium	Hunger medium containing 0.05 mCi "Pro-mix"/ml
SF-900 II medium	"Sf-900 II SFM Basal Powdered Medium" containing "Sf-900 II Supplement", 0.35 g $Na(CO_3)_2$ and 10 % (v/v) FCS, pH 6.2
Timelab medium	MEM-Minimum Essential Medium containing Hanks' salts, L-glutamine, 25 mM HEPES, 0.35 g/l $NaHCO_3$, 4.5 g/l glucose and MEM non-essential amino acid solution, 10 % (v/v) FCS

3.1.5 Antibodies and primary antibodies:

anti-AbI 1 (clone 24-11) Santa Cruz Biotechnology, Heidelberg

anti-BrdU (clone B44), coupled to FITC Becton Dickinson, Hamburg

anti-Cullin 1 (Hs), rabbit antiserum Laboratory Willi Krek, Basel
directed against human Cullin1

anti-Cyclin A (T310), rabbit antiserum Hengst et al., 1994
directed against human cyclin

anti-cyclin B1 (clone GNS1) Santa Cruz Biotechnology, Heidelberg

anti-cyclin D1 (clone DCS-6) Laboratory Jiri Bartek, Kopenhagen

anti-cyclin E1 (clone HE12) Laboratory Ed Harlow, Harvard, USA

anti-Grb2, goat antiserum directed against AA 195-217 of human Grb2, Santa Cruz Biotechnology, Heidelberg

anti-Grb2 (clone 3F2) Upstate Biotechnology, Eching

anti-GST, rabbit antiserum directed against GST Laboratory of Ludger Hengst

anti-HA.11 (clone 16B12) BabCo, Richmond, USA

anti-Hsp70/Hsc70, rabbit antiserum, directed against Hsp70/Hsc70 Laboratory of Jörg Höhfeld, Bonn

anti-Lyn (clone 42) Becton Dickinson, Hamburg

anti-p19SKP1, rabbit antiserum, directed against human p19SKP1 Laboratory of Willi Krek, Basel

anti-p21 (clone CP36 and CP74, mixed) Upstate Biotechnology, Eching

anti-p21, rabbit antiserum directed against the last 19 C-terminal aa from human p21 Santa Cruz Biotechnology, Heidelberg

anti-p21, goat antiserum directed against p21 this study

anti-p27/Kip1 (clone 57) Becton Dickinson, Hamburg

anti-p27, rabbit antiserum directed against the last 19 C-terminal aa of human p27 Santa Cruz Biotechnology, Heidelberg

anti-p27 (G96) goat antiserum this study

directed against p27

anti-p27-pY88 this study

anti-p45^{SKP2}, rabbit antiserum Laboratory Willi Krek, Basel

directed against human p45^{SKP2}

anti-p57, rabbit antiserum directed against the last 20 C-terminal aa of human p57, Santa Cruz Biotechnology, Heidelberg

anti-PSTAIRE, monoclonal antibody directed against the PSTAIRE region of cyclin-dependent kinases Dulic et al., 1992

anti-phospho-p27, rabbit antiserum directed against human, on T187 phosphorylated p27 Upstate Biotechnologies, Eching

anti-phospho-p27, rabbit antiserum directed against human, on T187 phosphorylated p27 Zymed Laboratories, South San Francisco, USA

anti-phospho-tyrosine (clone 4G10) Upstate Biotechnologies, Eching

anti-phospho-tyrosine (clone P-Tyr-100) Cell Signaling Technology, Frankfurt am Main

anti-RB, goat antiserum directed against the last 15 C-terminal aa of human pRb Santa Cruz Biotechnology, Heidelberg

anti-Src, rabbit antiserum directed against human Src Santa Cruz Biotechnology Heidelberg

anti-α-tubulin (clone DM 1A) Sigma-Aldrich, Taufkirchen

Secondary antibodies:

goat anti-mouse IgG, (H+L), peroxidase-coupled, Pierce, Rockford, USA

goat anti-rabbit IgG, (H+L), peroxidase-coupled, Pierce, Rockford,

USA

anti-goat IgG, peroxidase-coupled Santa Cruz Biotechnology, Heidelberg

goat anti-mouse IgG, (H+L), FITC-coupled Jackson ImmunoResearch Laboratories, West Grove, USA

goat anti-rabbit IgG, (H+L), FITC-coupled Jackson ImmunoResearch Laboratories, West Grove, USA

goat anti-mouse IgG, (H+L), rhodamine-coupled Jackson ImmunoResearch Laboratories, West Grove, USA

goat anti-rabbit IgG, (H+L), rhodamine-coupled Jackson ImmunoResearch Laboratories, West Grove, USA

3.1.6 Enzymes

Enzymes were obtained from Calbiochem-Novabiochem GmbH (Bad Soden), New England Biolabs (Schwalbach/Taunus), MBI Fermentas (St. Leon-Rot), Promega (Mannheim), QIAGEN (Hilden), Roche (Mannheim) and Stratagene (Heidelberg). If not stated otherwise, the reaction conditions given by the manufacturer were used.

3.1.7 DNA Oligonucleotides

DNA oligonucleotides were obtained from MWG-Biotech (Ebersberg) and Metabion (Martinsried).

Primer DNA oligonucleotides for the amplification and mutagenesis of p27 and

p27 fragments:

Primer	DNA sequence
LHSD37	5'-GGATCCGGGAGACATATGTCAAACGTGCG-3'
LHSD40	5'-GGAGTCTTCTGCAGTTTGCATTACTATCCCTAGG-3'
Mg1-p27LF/AA	5'-GCCTGCAGGAACGCCGCCGGC-3'
Mg2-p27FY/AA	5'-CCGCGGGGGTCTGTAGGCGGCCTCG-3'
Mg6-p27-Nt	5'-CACCTTGAATTCTCATTTGGGGGGCC-3'
Mg7-p27-Ct	5'-CGCGGCCCCATATGGGTGCCTGCAAG-3'
p27dSH3	5'-CCCGCGGGGCGGCGCAGGTGCCTGCAAGGTG-3'
p27-F/Y2, Re-FD	5'-GCTTGCCCGAGTTCTATTTCAGACCCCCGCGG -3'
p27-F/Y2, Re-Rev	5'-CCGCGGGGGTCTGAAATAGAACTCGGGCAAGC -3'
p27-F/Y3, Re-FD	5'-GCTTGCCCGAGTTCTTCTACAGACCCCCGCGGCC -3'
p27-F/Y3, Re-Rev	5'-GGCCGCGGGGGTCTGTAGAAGAACTCGGGCAAGC -3'
p27-N SH3-TGA	5'-GGGTCAGTAGTAGAACTCG -3'
p27-Y1 FD	5'-CCCCTAGAGGGCAAGTTCGAGTGGCAAGAG -3'
p27-Y1 Rev	5'-CTCTTGCCACTCGAACTTGCCCTCTAGGGG-3'
p27-Y2Y3B	5'-GCCGCGGGGGTCTGAAGAAGAACTCGG -3'

Primer DNA oligonucleotides for the amplification and mutagenesis of p21 and p21 fragments:

Primer	DNA sequence
p21-Ct-ATG-NdeI	5'-CTACCTTCATATGGGGCCC -3'
p21 Stop-Rev	5'-GGCGGATTAGGGCTTCCTC -3'
p21Y1/F-FD	5'-GCCTGCCCAAGCTCTTCCTTCCCACGGGGCC -3'

p21Y1/F-Rev	5'-GGCCCCGTGGGAAGGAAGAGCTTGGGCAGGC -3'
p21Y2/F-FD	5'-GCATGACAGATTTCTTCCACTCCAAACGCCG -3'
p21Y2/F-Rev	5'-CGGCGTTTGGAGTGGAAGAAATCTGTCATGC -3'

Primer DNA oligonucleotides for the amplification of Grb2:

Primer	DNA sequence
GRB2-ATG	5'-CTCACCATGGAAGCCATC -3'
GRB2-TAA	5'-CTTGACTCTTAGACG -3'

Primer DNA oligonucleotides for the amplification of c-Abl and of c-Abl fragments:

Primer	DNA sequence
Abl-inc SH3	5'-CATATGCCCAACCTTTTTGTGGCA -3'
Abl Kin.Dom.- ATG	5'-CATATGGTCAACAGCCTGGAGAAAC -3'
Abl Kin.Dom.- TGA	5'-CTTCAGCAGGTTCTGGTCTTGGTG-3'
Abl-NdeI-Exon IB	5'-GCATATGGGGCAGCAGCCTGG -3'
Abl-TAG	5'-GCAGGCGGATCCGACGGGCC -3'

3.1.8 Plasmids

pApuro	Laboratory of Markus Warmuth, Hämatologikum der GSF, Großhadern
pBluescript SK (-)	Stratagene, Heidelberg
pCMX.pl2	Laboratory Roland Schüle, Freiburg
pCR2.1	Invitrogen, Groningen,

Netherlands

pCRII Invitrogen, Groningen, Netherlands

pCruz-HA Santa Cruz, Heidelberg

pEGFP-C2 CLONTECH Laboratories, Heidelberg

pET-3a,-11a, -24d, -28a Calbiochem-Novabiochem GmbH, Bad Soden

pEYFP-C1 CLONTECH Laboratories, Heidelberg

pGEM-T, -T Easy Promega, Mannheim

pGEX-2T, -2TK, -5X1 Amersham Pharmacia Biotech, Freiburg

prk5 Laboratory of Axel Ullrich, „MPI für Biochemie“, Martinsried

Cell lines

HeLa, suspension cells human cervical carcinoma cell line

HeLa, adherent cells human cervical carcinoma cell line

HS68 human diploid fibroblasts from newborn foreskin, ATCC-number CRL-1635

293T cells human embryonic kidney 293 (ATCC-number CRL-

1573)	
	additionally transformed with
the	
	large T-antigen of the SV-40
virus	
MCF7	adherent human mammary
gland	
	epithelial cells adenovirus-transformed (ATCC-number HTB-22)
Sf9	ovary cell line from the pupa
of	
	Spodoptera frugiperda, strain IPLB-Sf21AE

3.2 Molecular biological methods

3.2.3 Preparation of plasmid DNA

Plasmid DNA was prepared according to the manufacturer's instructions using kits from Qiagen, Hilden, Germany.

3.2.10 Directed *in vitro* mutagenesis

Directed mutagenesis was carried out according to the manufacturer's instructions using the QuickChange Site-Directed Mutagenesis Kit from Stratagene, Heidelberg, Germany.

3.3 Cell biological methods

3.3.1. Culturing human cell lines

Adherent cell lines (HeLa, 293T, HS68 and MCF7) were cultured in cell culture dishes of variable size in standard medium at 37°C, 5 % CO_2 and 100 % air humidity (Hera cell, cell culture incubator, Heraeus, Hanau). 1 mM sodium pyruvate was

additionally added to the medium for HS68 cells. HeLa, MCF7 and 293T cells were passaged every three days before confluence was reached. For this the cells were firstly washed with PBS, detached from the culture dish with 10 x trypsin/EDTA and sown out at a dilution of 1:3 to 1:6 on new culture dishes. Cell counts were in general determined by means of a Neubauer counting chamber. HS68 cells were cultured under contact inhibition for several days to several weeks during which the medium was replaced every three days. If required the cells were treated with trypsin as described and sown in new culture dishes at a dilution of 1:4 to 1:8. HeLa suspension cells were cultured in Joklik's medium at 37°C in spinner flasks while stirring constantly. The cell density of the cultures was kept between 2.5 x 10^5 and 10^6 cells per ml by daily dilution with fresh medium.

3.3.2. Culturing insect cells

Sf9 insect cells were used for the baculovirus-mediated overexpression of recombinant mammalian proteins. These were cultured in suspension culture in Sf-900II SFM medium at a cell density of 5 x 10^5 cells per ml in a shaking incubator (innOva4230, New Brunswick Scientific GmbH, Nürtingen) at 80-90 rpm and 27°C. The cells were diluted with fresh medium at a cell density of more than 2 x 10^6 cells per ml. Insect cells were infected at a low infection rate per cell (< 1) in order to amplify the baculovirus and the supernatant of the lysed cells was harvested and centrifuged (10,000 g, 5 min) after 4-5 days. In order to obtain a suspension with a high virus titre, the supernatant was again added to insect cells and cultured until lysis of the cells. This procedure was repeated two to three times. The clear centrifuged supernatant with a high virus titre was used directly for protein expression or stored at 4°C for up to 6 months. 2 x 10^6 suspension cells per ml were sown out for the protein expression and inoculated with the supernatant of the lysed Sf9 cells with a high titre to achieve about three to ten virus infections per cell. The infected insect cells were cultured for a further three to four days at 27°C, they were centrifuged for harvesting (2500 rpm, 5 min), washed with PBS, again centrifuged, frozen in liquid nitrogen and stored at -80°C. A plaque assay was carried out to

determine the virus titre of a solution. For this Sf9 insect cells were cultured adherently on tissue culture dishes and incubated for 1 h at 27°C with various dilutions (10^{-3}-10^{-5}) of the virus solution. The Sf9 cells were subsequently overlayered with 1 % low-melting agar (Agarplaque-plus agarose) in Sf-900 II SFM medium in order to avoid diffusion of the virus during cell lysis. After 6-10 days incubation at 27°C the infected cells were lysed depending on the virus titre so that the concentration of the virus particles in a solution could be deduced from the number of holes that were formed in the Sf9 cell lawn.

3.3.7 Real-time microscopy and microinjection

Adherent HeLa tissue culture cells were examined by microscopy either directly or after transient transfection for several hours to several days using an Olympus IX70 (Olympus, Hamburg) reverse microscope with an ST-133 controller (Roper Scientific, USA) and a polychrome IV filter (Photonics, Martinsried), remodelled with a 37°C incubation chamber (Solent Scientific Limited, Portsmouth, England). For this the cells were transferred to a special Timelab medium 24 h before the microscopy. This medium enables a culture of tissue culture cells that is independent of the CO_2 cell incubator.

In order to investigate the location of a protein and to determine protein-protein interactions and thus any associated changes in the location of a protein, cells were transfected with a GFP- or YFP-fusion construct of the respective protein under examination. The cells were either already transfected with the corresponding DNA before the real-time microscopy or cotransfected with the DNA of the interaction partner, or DNA (50 µg/µl) or protein (1-5 mg/ml) was specifically delivered into the cells by microinjection (FemtoJet and InjectMan, Eppendorf, Hamburg). The cells were photographed at 1 min to 1 h intervals in the phase contrast channel and identically in the fluorescence channel using the microscopy and evaluation software IP-Lab version 3.5.4 for Macintosh (Visiton Systems, Puchheim). The individual

phase contrast and fluorescence images were superimposed using this program and the individual pictures were compiled to form a continuous film.

3.4 Biochemical methods

3.4.2 Immunological and immunobiochemical methods

3.4.2.2 Immunoprecipitation of proteins

Precipitations with specific antibodies were carried out in order to purify a certain protein from total cell extracts (pulse-chase labelling, *in vitro* translation preparations or interaction investigation) or in order to identify the proteins associated *in vivo* with the precipitated protein in coimmunoprecipitations. All steps were carried out at 4°C or on ice using precooled solutions.

The specific antibody (1-3 µg) was bound to 20 µl 50 % /v/v) protein-A-Sepharose or 20 µl 50 % (v/v) protein-G-Sepharose (1 h on a overhead rotator). After washing twice in IP buffer the suspension was incubated for 1 – 3 h with the protein extract in order to form the antigen-antibody complexes. After washing again three times with IP buffer, the Sepharose beads were taken up in 25 µl 2 x SDS sample buffer and analysed after 5 min boiling by SDS-PAGE and Western blot.

3.4.2.4 Preparation of antisera and affinity purification of antibodies

In order to prepare the antigen the respective proteins were expressed recombinantly in E. coli as a GST or His fusion and purified by diverse methods up to the highest possible homogeneity. The purified protein (1 mg/ml) was intensively emulsified for the first injection in a ratio of 1:1 with TiterMax Gold using a double cannula. 250 µg protein was injected subcutaneously to immunize rabbits and 500 µg protein was used for goats. After 6 weeks further injections were carried out at intervals of 14 days in which TiterMax Gold was replaced by Freund's adjuvant. The withdrawn blood was kept for 2 h at room temperature for clotting and was subsequently centrifuged for 15 min at 5500 rpm to obtain the serum. The serum was stored at -20°C or used directly for the affinity purification of antibodies. For the affinity

purification of the specific antibody, 2-4 mg of the antigen was coupled covalently to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions and washed. The serum was diluted 1:1 with PBS and incubated overnight at 4°C with the immobilized antigen on an overhead rotator. The Sepharose was washed five times with 0.5 M NaCl in PBS and transferred to a column. The antibody was eluted with 0.2 M acetic acid, 0.5 M NaCl. The column eluate was collected in 0.5 ml fractions and immediately neutralized with 100 µl 1 M Tris, pH 9.0 per fraction. The individual fractions were tested by spectral photometry for their antibody content. The fractions containing antibody were pooled, dialysed against PBS and concentrated (UH 100/l ultracartridge apparatus, Schleicher & Schüll, Dassel). Gelatin and sodium azide was added to the antibody solution to a final concentration of 0.2 % (w/v) and 0.1 % (w/v). The quality of the purified antibody was tested in an immunoblot using a concentration titration of the antibody against the specific antigen and total cell extract of HeLa cells. Phospho-specific antibodies against p27 were raised after synthesis of peptides where the desired phospho-amino acid was incorporated into the peptide. For tyrosine residue 88 the peptide NH_3-CLPEF(Y-PO_4)YRPPR-COO^- was synthetised, where (Y- PO_4) is phosphotyrosine. Similar antisera were raised against the other tyrosines in p21, p27 and p57. Peptides were coupled to ovalbumin and injected intoi mammals (rabbits. goats and mice). Polyclonal antisera were purified first by depleting of all antibodies that cross-reacted with the non-phosphorylated peptide and subsequently by affinity purification against the phosphopeptide.

3.4.3. 2-Dimensional gel electrophoresis

All materials for isoelectric focussing were obtained from Amersham Pharmacia Biotech (Freiburg). The focussing was carried out in a cooled (20°C) Multiphor II according to the manufacturer's instructions. Isoelectric focussing strips (Immobiline DryStrip NL 3-10 or pH 6-11) containing 0.1 mg – 2 mg protein in 300 µg rehydration buffer (8 M urea, 2 % (w/v) CHAPS, 2 % (v/v) IPG buffer (pH 3-10 or pH 6-11), 18.5 mM DTT) were incubated overnight in a rehydration cassette covered

with a layer of oil for the focussing. The strips were subsequently focussed in a Multiphor II with an increasing voltage gradient.

Gradient	1st phase 300 V, 30 min	6th phase 2200 V, 30 min
	2nd phase 600 V, 30 min	7th phase 2700 V, 30 min
	3rd phase 900 V, 30 min	8th phase 3200 V, 30 min
	4th phase 1200 V, 30 min	9th phase 3500 V for 18.5 to 27 h
	5th phase 1700 V, 30 min.	

They were focussed for a total of 70,000 – 100,000 V/h depending on the amount of protein. The strips were briefly rinsed with ddH_2O, frozen at -80°C or used directly for the separation in the second dimension. For this the strips were incubated three times for 5 min in equilibration buffer I (6 M urea, 30 % (v/v) glycerol, 2 % (w/v) SDS, 50 mM Tris/HCl pH 8.8, 10 mg/ml DTT), again briefly rinsed with ddH_2O and incubated three times for 5 min in equilibration buffer II (identical to equilibration buffer I but containing 25 mg/ml iodoacetamide instead of DTT). The strips treated in this manner were briefly rinsed with ddH_2O, drained and poured over an SDS-PAGE separation gel (12 %, 20 x 20 cm) with hot 0.5 % (w/v) agarose, dissolved in SDS electrophoresis buffer with traces of bromophenol blue. Electrophoresis in the second dimension was carried out as described under section 3.4.1.2. The separated proteins were analysed by Coomassie or silver staining or by specific antibodies after SDS-PAGE and immunoblot.

3.4.4 Preparation of total cell lysates

Suspension HeLa cells were used to prepare total cell lysates from tissue culture cells or adherent cells were detached from the substrate with trypsin, centrifuged for 2 min at 1500 rpm and washed twice with PBS. The cell pellet was resuspended in IP or RIPA buffer and lysed by ultrasound on ice (Labsonic U, ultrasonic homogenizer, B. Braun, Melsungen). Alternatively IP or RIPA buffer was added directly to tissue

culture cells after washing twice with PBS and they were transferred into the buffer by scraping them off with a cell scraper. Also in this case they were lysed by ultrasonic treatment. The crude extract was subsequently centrifuged for 15 min at 13000 g and 4°C.

3.4.5 Metabolic labelling of tissue culture cells with [^{32}P] *ortho*-phosphate and thin layer chromatographic separation of phosphoamino acids

A 250 ml flask of adherent HeLa or MCF7 tissue culture cells was cultured in each case up to a confluence of 80 % for the metabolic labelling. These were washed twice with warm TBS and once with phospholabel medium and subsequently cultured for a further 4 h with 0.5 mCi [^{32}P] *ortho*-phosphate per ml phospholabel medium. The tissue culture cells were washed three times with PBS, 1 ml IP buffer (containing 150 µM Na ortho-vanadate and 100 µM KF phosphatase inhibitors) was added and they were detached from the culture substrate with a cell scraper. The cell suspension was homogenized on ice using a G23 cannula, boiled for 10 min at 100°C, cooled for 10 min on ice and centrifuged for 10 min at 13000 g. The supernatant was incubated with 50 µl protein A-Sepharose (50 %, (v/v)) for 1 h on an overhead rotator at 4°C in order to bind unspecific proteins. The binding to the precoupled antibody was also carried out for 1 h at 4°C. The Sepharose beads were washed four times with IP buffer containing phosphatase inhibitors and taken up in 25 µl 2 x SDS sample buffer, separated by SDS-PAGE and blotted. After amido black staining the PVDF membrane was exposed to a sensitive film (MS) for one to two days using an amplifying screen. The resulting radioactive signals were matched with the membrane stained with amido black and the radioactively labelled proteins were cut out of the PVDF membrane. In this process only two thirds of a band was cut out in each case in order, after exposing the membrane again, to check whether the cut out piece of membrane corresponds with the radioactive signal. An antibody detection was also carried out with the remaining membrane in order to allocate the cut out bands to the desired specific protein.

The cut out piece of membrane was rehydrated briefly in methanol, washed twice in ddH_2O and 25 µl 6 N HCl was added. The protein was hydrolysed for 50 min at 110°C in an incubator. The reaction was stopped with 25 µl ddH_2O and centrifuged. The supernatant was transferred to a new 0.5 ml reaction vessel and dried in a MD8C membrane vacuum pump (Vakuubrand, Wertheim). The dried hydrolysate was resuspended in 3.5 µl TLC buffer I, 1 µl phosphoamino acid standard (phosphoserine, phosphothreonine and phosphotyrosine, 0.3 µg/ml of each, Sigma) was added and 0.25 µl fractions were applied in one point on a thin layer chromatography cellulose plate. The phosphoamino acids were separated electrophoretically in two dimensions using a HTLE-7000 apparatus (CBS Scientific, USA) according to the manufacturer's instructions. The first dimension was carried out for 35 min at pH 1.9 in TLC buffer I at 1.5 kV. The plate was subsequently dried for 20 min with a hairdryer and a second electrophoresis rotated by 90° was carried out with TLC buffer II for 30 min at pH 3.5 and 1.3 kV. The plate was dried at 60°C and the phosphoamino acid standard was visualized with 0.5 % ninhydrin solution in acetone at 80°C. The radioactive phosphoamino acids were detected by a one to three week exposition of the TLC plate with an amplifier screen against a sensitive MS X-ray film.

3.4.7 Determination of the half-life of a protein by metabolic labelling with [^{35}S] methionine and [^{35}S] cysteine (pulse-chase)

In order to analyse the half-life of p27 and derived mutants, a 15 cm cell culture dish of HeLa tissue culture cells (80 % confluent) was transfected in each case with HA fusion constructs of p27, they were detached from the culture dish with trypsin 5 h after the transfection and divided in equal portions per construct among four fresh cell culture flasks (corresponding to the number of the planned times for the half-life determination of the protein) and the culture was continued overnight. The cells were washed once with PBS and cultured for 60 min with 5 ml Hunger medium per flask to reduce endogenous methionine and cysteine.

"Pro-mix L-[^{35}S] in vitro cell labelling mix" (Amersham) at a final activity of 0.05 mCi/ml [35S] methionine and [^{35}S] cysteine was subsequently added to the cells and they were metabolically labelled for 1 h. The radioactive medium was in each case replaced by 25 ml chase medium, the medium was immediately removed again in the case of the 0 h value and the cells were harvested. For the other times the labelled cells were cultured for a further 2 h, 4 h and 6 h and subsequently harvested. For this the cells were washed with PBS, 1 ml 10 x trypsin was added and they were incubated for 2 min at 37°C. 10 ml chase medium was added to the detached cells, they were transferred to a 15 ml Falcon vessel, centrifuged for 2 min at 1500 rpm, resuspended in 1.5 ml ice-cold PBS and transferred to a 2 ml reaction vessel. After a renewed centrifugation (2 min, 1500 rpm) the cell pellet was frozen in liquid nitrogen and stored at -80°C.

The cells were lysed in 750 µl IP buffer in each case by homogenization with a G23 cannula on ice. The samples were centrifuged for 5 min at 13000 g and the protein content of the supernatant was determined with the "Micro BCA Protein Assay Reagent Kit". The supernatant was transferred to a 1.5 ml reaction vessel, boiled for 10 min at 100°C, cooled for 10 min on ice and the precipitated proteins were pelleted by centrifugation (10 min, 13000 g). In each case exactly identical amounts of protein were incubated with identical amounts of protein G-Sepharose-bound HA antibody for 1 h at 4°C, washed three times with lysis buffer, centrifuged and taken up in 25 µl 2 x SDS sample buffer. The samples and aliquots of the supernatants were separated by SDS-PAGE and subsequently analysed by Coomassie staining and auto-radiography. The [^{35}S] total incorporation and the half-life of p27 was quantified with a Fujifilm BAS-2500 Phosphoimager (Fujifilm, Düsseldorf).

3.4.8 Expression of recombinant proteins in *E. coli*

In order to express recombinant proteins the corresponding plasmid constructs were transformed in the *E. coli* strain BL21(DE3) and an individual colony was inoculated in 200 ml to 10 l Superbroth medium. Protein expression was induced with 1 mM

IPTG in the exponential growth phase at an OD600 of 0.8. After 4 to 5 h at 20°C (e.g. the kinases Abl^{KD}, Abl^{KD+SH3}), 30°C (the isolated SH3 domains and CAKp1-GST) or 37°C (all other proteins expressed in this study) the cells were centrifuged, washed once with PBS and directly processed further or stored at -20°C.

3.4.9 Native and denaturing affinity purification of GST and His fusion proteins, renaturation of proteins

In this study proteins were produced recombinantly in *E. coli* as GST or His fusion proteins and purified therefrom. Two basic methods were used to purify proteins. If the protein was present in a soluble form in E. coli, a native affinity purification of the protein was carried out by means of a nickel-chelate or Glutathione Sepharose matrix. If the recombinant protein was present in an insoluble form in bacteria (inclusion bodies), a denaturing purification with subsequent renaturation of the protein was carried out.

Cell lysis:

The bacterial pellet was taken up in resuspension buffer (200 mM NaCl, 25 mM Na phosphate pH 8.0, 2.5 mM β-mercaptoethanol, 1 mM PMSF, 10 µg/ml aprotinin, pepstatin A, leupeptin) and disrupted on ice by means of ultrasound (Labsonic U, ultrasonic homogenizer, B. Braun, Melsungen). As an alternative to the ultrasonic treatment the bacterial pellet was lysed with 3.5 mg/ml lysozyme for 30 min at room temperature in 150 mM NaCl, 50 mM Tris pH 8.0 in the case of particularly sensitive proteins (e.g. the kinases Abl^{KD}, Abl^{KD+SH3}).

Native purification of proteins by nickel-chelate of glutathione Sepharose affinity chromatography:

A native purification by nickel-chelate chromatography was used for Abl^{KD}, Abl^{KD+SH3}, p27 and for the shortened form of p27 and amino acid substitutions of p27, p57 and for Grb2-His. The disrupted bacterial cells were centrifuged for 35 min at 45000 rpm to separate the bacterial cell debris. Approximately 1 mg "ProBond

Nickel-Chelating Resin" per 10 mg recombinant protein was added to the centrifugation supernatant and incubated for 1 h at 4°C on an overhead rotator. The immobilized protein was washed three times with resuspension buffer (additionally containing 10 mM imidazole) and eluted with 300 mM imidazole. The eluate was dialysed, optionally concentrated (Centricon, centrifuge filter units, ultrafiltration with Amicon ultrafiltration cell) or, if a higher degree of purity of the proteins was necessary, subjected to further purification steps.

The purification of GST fusion proteins was carried out similarly by means of a glutathione Sepharose matrix. In this case the immobilized protein was eluted with 20 mM glutathione at pH 8.0. Glutathione purification was used for the isolated SH3 domains of the 41 signal transduction proteins and for the isolated domains of Grb2, for p27-GST, Grb2-GST, for CAKp1-GST and for GST itself.

Denaturing purification of proteins by nickel-chelate or glutathione Sepharose affinity chromatography:

In order to purify inclusion bodies (e.g. p21 and amino acid substitutions of p21, FBX3) the pellet was resuspended by ultrasonic treatment in 200 mM NaCl, 50 mM Tris/HCl pH 8.0, 10 mM EDTA and 0.5 % (w/v) Triton-X-100 solution to solubilize the bacterial membranes, incubated for 15 min at room temperature on an overhead rotator and centrifuged for 15 min at 10000 rpm and room temperature. This procedure was repeated twice, the pellet was subsequently homogenized with ultrasound in resuspension buffer, washed and centrifuged for 15 min at 10000 rpm. The inclusion body pellet was dissolved overnight at room temperature in denaturation buffer (8 M urea, 0.1 M Na phosphate, pH 8.0) in order to denature the protein. In the case of very poorly soluble proteins ($Src^{rec.}$) the pellet was dissolved with 12 M urea at 50°C for 4 h. The denatured protein solution was centrifuged at 10000 rpm for 15 min at room temperature.

The supernatant was applied to a nickel-chelate matrix, preequilibrated with denaturing buffer and incubated for 1-2 h on an overhead rotator. The immobilized proteins were washed three times with denaturing buffer and eluted with 300 mM imidazole in denaturing buffer.

The purification of GST fusion proteins (cdc14 active and cdc14 inactive) by a Glutathione Sepharose matrix was carried out analogously. The concentration of the urea solution was adjusted to 4 M after the proteins dissolve. The fusion protein was eluted from the glutathione Sepharose matrix with 20 mM glutathione at pH 8.0.

Renaturation of affinity-purified denatured proteins:

Four different renaturation strategies were used to renature the denatured affinity-purified proteins depending on the respective protein. In the simplest case the eluted protein could be refolded by a step dialysis (p57 from inclusion bodies). For this the eluate was dialysed in a dialysis tube for 2 h in each case with a urea concentration which was reduced by 2 M in each step. The last dialysis step was against IP buffer. Alternatively the protein was left immobilized on the chelate or glutathione matrix, renatured there with the same urea solutions of decreasing concentration and subsequently eluted from the matrix (cdc14) with imidazole (300 mM, pH 8.0) or glutathione (20 mM, pH 8.0). In another renaturation strategy the denatured protein eluate was diluted to 15 times its original volume by the dropwise addition of IP buffer (using a membrane pump for 24 h) while stirring continuously. Precipitated proteins were centrifuged and the supernatant was dialysed again overnight against IP buffer in order to remove the remaining urea ($Src^{rec.}$). The case of proteins that are very difficult to renature (p21 and amino acid substitutions of p21) ion exchange chromatography with immobilized renaturation and subsequent elution of the renatured protein by a salt gradient was used (see section 3.4.10). If necessary, the renatured proteins were concentrated (Centricon, centrifuge filter units, ultrafiltration with Amicon ultrafiltration cell) or, if a higher degree of protein purity was required they were subjected to further purification steps.

3.4.10: Ion exchange chromatography and gel filtration

Ion exchange chromatography

Chromatography on anion or cation exchangers was used to purify or concentrate proteins (p27 and derived forms, p57). Protein solutions were used for this from previous native or denatured affinity purifications (p57 from inclusion bodies), (see 3.4.9). Ion exchange chromatography was also used to renature proteins (p21 and derived forms). The chromatographies were carried out using anion and cation exchanger columns from Amersham. The chromatography was carried out with an "Äktapurifier FPLC/HPLC" system using the column parameters (program UNICORN 3.00) specified by the manufacturer (Amersham Pharmacia Biotech, Freiburg) for the respective column. In order to purify native proteins, the protein solution was dialysed for 24 h against a buffer having a low salt content (25 mM NaCl, 25 mM Tris pH 8.0, 1 mM PMSF, 1 mM DTT; buffer A). The protein solution was loaded onto an ion exchanger column preequilibrated with buffer A and non-bound proteins were washed out with 10 column volumes of buffer A. The protein was eluted from the column with a gradient of buffer A to buffer B (0.5 M – 1 M NaCl (depending on the protein), 25 mM Tris/HCl pH 8.0, 1 mM PMSF, 1 mM DTT) at a flow rate of 1 ml/min. The eluate was collected in 0.25 ml fractions and analysed after SDS-PAGE by Coomassie staining or in an immunoblot.

In order to renature proteins, the denatured protein was dialysed for 24 h against denaturation buffer (8 M urea, 25 mM NaCl, 25 mM Tris, pH 8.0) and applied to a column preequilibrated with denaturing buffer. The column was washed with 10 volumes of denaturing buffer to remove unbound protein. The protein bound to the column matrix was renatured with a very slow gradient (0.1 ml/min flow rate) of denaturing buffer to buffer A over 24 h. The protein was eluted with a second gradient of buffer A to buffer B. The fractions of the desired protein were pooled, concentrated and frozen or optionally further purified.

Gel filtration:

Further purifications of proteins after an affinity and ion exchange chromatography were carried out by gel filtration of the protein sample (p27 and derived forms, p21 and p57). In addition analytical gel filtration was used to analyse the composition of a protein complex and to estimate the size of proteins in solution (CDK2/cyclin A; CDK2/cyclin A/p27; p27-Grb2). Gel filtrations were carried out with prepacked gel filtration columns (Amersham) using the FPLC/HPLC-Äktapurifier system. A protein solution was applied to a column prequilibrated with elution buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1 mM PMSF, 1 mM DTT) and fractionated according to size in an isocratic elution over 1.5 column volumes.

The column eluate was collected in 0.25 ml – 5 ml fractions and analysed after SDS-PAGE by Coomassie staining or in an immunoblot.

3.4.11 Purification of Cip/Kip proteins by boiling and stabilizing proteins

A specific purification strategy for Cip/Kip proteins is based on the thermal stability of inhibitor proteins. Mammalian cell extracts or protein extracts from bacterial expressions of the recombinant inhibitors were boiled for 10 min at 100°C, cooled for 10 min on ice and separated from the precipitated protein impurities for 35 min at 45000 rpm. The resulting protein solution was used directly for investigations or the desired was further purified.

The proteins purified with the described methods (3.4.8-3.4.11) were usually immediately frozen in liquid nitrogen and stored at -80°C. In the case of very unstable proteins, the proteins were stabilized with stabilizing reagents (1-10 % (w/v) glycerol (AblKD, AbIKD+SH3, CDK2/cyclin A), with 1 mg/ml BSA (cdc14, Srcrec.) or with 100 mM arginine for p21 and derived forms). In addition proteins were stabilized by varying the salt concentrations or the pH of the storage buffer.

3.4.12 Purification of recombinant proteins from insect cells

Active cyclin A-CDK2 from overexpressing insect cells was purified by nickel-chelate affinity purification and subsequent gel filtration. If not stated otherwise, all purification steps were carried out at 4°C or on ice and with precooled solutions. Human cyclin A and an amino-terminal hexahistidine fusion protein of human CDK2 were expressed in Sf9 with the aid of recombinant baculoviruses. The baculovirus constructs originated from the laboratory of Dr. D. Morgan (University of California, San Francisco, USA).

The two insect cell pellets were resuspended in an equal volume of baculovirus lysis buffer (40 mM Na phosphate, 600 mM NaCl, 20 % (w/v) glycerol, pH 8.0, 0.1 mM DTT, 2 mM PMSF, 2 x complete without EDTA (Roche), 20 mM $MgCl_2$, 8 mM ATP) and disrupted with the aid of a dounce homogenizer (Braun, type L). The homogenisate for CDK2 was admixed with recombinant CAK1p protein (10 µg of a kinase activating kinase from yeast that can phosphorylate CDK2) and incubated for 45 min at 30°C for phosphorylation and activation by the endogenous kinases of the insect cells and the recombinant CAK1p (Kaldis, 1999). The two homogenisates were combined and incubated for 1 h to form the CDK2/cyclin A complex. The homogenisate was centrifuged for 35 min at 35000 rpm, Probond nickel-chelaing resin was added to the supernatant and it was incubated for 2 h at 4°C on an overhead rotator to immobilize the cyclin A-CDK2 complex. The complex was washed four times in IP buffer containing 10 mM imidazole and eluted from the chelate matrix with 300 mM imidazole in IP buffer. For further purification the eluate was concentrated further over a Superdex 200 pg HiLoad 26/60 gel filtration column (elution buffer: 150 mM NaCl, 50 mM Tris/HCl pH 7.5, 1 mM DTT, 0.5 mM PMSF). After analysis of the gel filtration fractions by SDS-PAGE and Coomassie staining, the fractions containing the cyclin A-CDK2 complex were frozen in liquid nitrogen and stored at -80°C.

3.4.13 In vitro phosphorylation of proteins

In vitro phosphorylation experiments were used to identify the substrates of kinases, to locate phosphorylatable amino acid residues in proteins and to test the activity of proteins which inhibit the kinases in a coupled kinasation inhibition experiment. In the case of phosphorylation of substrate proteins the phosphorylation of serine/threonine residues as well as the phosphorylation of tyrosine residues was examined. Serine/threonine phosphorylations were carried out in kinasation buffer I. For this two mixtures were prepared per experiment which were combined for the kinasation:

mix A: 1 µl substrate protein (1-2 µg/µl)
1 µl ATP (25 µM final concentration)
add 10 µl ddH_2O

mix B: 1 µl kinase (different concentrations
1 µl kinasation buffer I (10 x)
0.5 – 1 µl [γ-^{32}P]ATP (10 µCi/µl)
add 10 µl ddH_2O

p21, p27 and derived forms thereof and histone H1 and pRb were used as substrate proteins. CDK2/cyclin A was used as the kinase.

The kinase reaction was carried out for 30 min at 30°C and stopped by adding 20 µl 2 x SDS sample buffer. The phosphorylated proteins were separated by SDS-PAGE and subsequently analysed by autoradiography. The activity of kinase inhibitors (p21, p27, p57 and forms derived therefrom) were tested in an analogous manner. In this case the inhibitor protein (1 µl) was added to mix A. In order to determine the Km of the inhibitor different amounts of the inhibitor protein (0.1 ng – 1 µg) were titrated against a constant amount of a kinase. Tyrosine phosphorylations were carried out in 20 µl kinasation buffer II. 0.5 – 2.5 µl kinase (Abl, Abl^{KD}, Abl^{KD+SH3}, Src^{Sf9}, $Src^{rec.}$ and Lyn; various concentrations), 1 µl substrate protein (0.5-1 µg/µl, p21, p27, p57 and forms derived therefrom, and Sam68, RanGAP1 and BSA), ATP (50 µM final concentration) and 0.5-1 µl [γ-^{32}P] ATP (10 µCi/µl) and 1 µl BSA (2 µg/µl) were incubated for 2 h at 30°C. The kinasation was stopped with 20 µl 2 x

SDS sample buffer and the phosphorylated proteins were separated by SDS-PAGE and detected by autoradiography.

Serine/threonine and tyrosine kinasations were also carried out in an immobilized form. For this either the kinase or the substrate was bound to glutathione or nickel-chelate Sepharose or one of the two components (substrate or kinase) was immunoprecipitated from the cell extract on protein A Sepharose using specific antibodies. The reaction itself was carried out under the conditions required for the respective phosphorylation (see above). For this 25 µl of the required kinase buffer was added to the components immobilized on the Sepharose matrix. The sample was mixed every 5 min in order to improve the intermixing of the immobilized kinasation reactions.

In order to quantify the radioactivity incorporated into a protein, the gel was dried after SDS-PAGE and Coomassie staining and the Cherenkov radiation of the cut out protein bands was measured. Alternatively the activity was determined by a Phosphoimager System (Fujifilm BAS-2500 Phosphoimager, Fujifilm, Düsseldorf).

3.4.14 In vitro protein-protein binding studies

For the binding studies one protein component was added first in a recombinant form as a GST or His fusion and the other component was then added in the form of *in vitro*-translated proteins or recombinant protein without a fusion tag. Both components were incubated in IP buffer for 1-3 h in an overhead rotator at 4°C, subsequently glutathione Sepharose or ProBond nickel-chelating resin was added and they were incubated for a further 30 min to 1 h. The immobilized complex was washed three times with IP buffer, centrifuged and taken up in 25 µl 2 x SDS sample buffer. Protein-protein interactions were analysed by SDS-PAGE and Coomassie staining, immunoblot analysis or autoradiography.

3.4.15 Identification of proteins by MALDI-TOF

In order to identify proteins the corresponding bands were cut out of the Coomassie-stained SDS-PAGE gels and directly digested in the gel using trypsin or LysC according to Eckerskorn and Lottspeich (1990). The mass spectroscopic analysis of the peptide fragments was carried out using a Bruker Reflex III MALDI – time of flight mass spectrometer (Bruker-Franzen, Bremen) and was carried out by the working group of Prof. Dr. Friedrich Lottspeich ("MPI für Biochemie", Martinsried). The determined peptide masses were evaluated by database analyses using the program MSFIT (http://prospector.ucsf.edu).

4. Results

4.1 Generation and characterization of a CDK- and cyclin-binding-deficient mutant of p27

$p27^{Kip1}$ binds numerous different CDK complexes. In order to identify binding partners of $p27^{Kip1}$ which associate with p27 independent of CDKs and cyclins, a CDK- and cyclin-binding-deficient protein ($p27^{\Delta CKI}$) was generated. $p27^{\Delta CKI}$ was constructed on the basis of the known X-ray structural data of the complex $p27^{wt}$-CDK2/cyclin A (Russo *et al.*, 1996a,). For this two amino acids in the two binding sites of p27 having the highest interaction with the inhibitor for CDK2 and cyclin A were substituted in p27 by means of PCR (L32, F33, F87, Y88/A). Both proteins, $p27^{wt}$ and $p27^{\Delta CKI}$ were cloned into pET28a and expressed recombinantly in *E. coli* as N-terminal hexahistidine fusion proteins. The proteins were purified by a nickel-chelate matrix. An additional purification was carried out by ion exchange chromatography (HighTrapSP, p27 elutes at 100 – 150 mM NaCl). In a subsequent step the fractions from the ion exchange chromatography containing p27 were purified on a gel filtration column (Superdex 200 pg HiLoad 26/60) up to a purity of > 96 %. The absence of inhibitory activity in $p27^{\Delta CKI}$ was demonstrated *in vitro* in a histone H1 kinase assay. In addition an examination by real-time microscopy showed that an N-terminal YFP fusion of $p27^{\Delta CKI}$ (pEYFP-C1) after transfection in HeLa cells no longer resulted in a cell cycle arrest. Cells transfected with

YFPp27$^{\Delta CKI}$ can progress through a complete cell division whereas HeLa cells transfected with p27^{wt}-YFP are arrested. This proves that p27$^{\Delta CKI}$ can no longer act as an inhibitor of the CDK complexes.

4.2 Identification of new binding partners of p27

p27^{wt}, p27$^{\Delta CKI}$, p21 and p57 were produced recombinantly in *E. coli* in large amounts (15 mg) and purified. p27 was purified as described in section 4.1. p21 was purified in a denaturing purification by nickel-affinity chromatography. The denatured protein was subsequently renatured on an ion exchange column (HighTrapSP) and eluted from the column with a salt gradient (p21 elutes at 400 – 450 nM NaCl). A purification strategy was designed for p57 using a native nickel affinity chromatography and an additional purification by ion exchange chromatography (HighTrapSP) at pH 8.0 and elution at 160-200 nM NaCl. p57 was finally gel filtered on a Superdex 200 pg HiLoad 26/60 column. The purified p27^{wt} and p27$^{\Delta CKI}$ were immobilized covalently on a matrix (Affi-Gel10).

p21 and p57 were covalently cross-linked with the affinity gel 10 matrix as a specificity control for the Cip/Kip proteins. These coupled proteins were incubated with a HeLa protein extract in IP buffer (from 25 l HeLa suspension culture in each case) in order to identify interaction partners of the immobilized proteins (figure 4.3). In a control experiment a pull down was carried out with p27^{wt}. In order to identify the expected CDKs and cyclins of this pull down, the interaction partners of p27 visualized in the Coomassie gel were cut out. The gel pieces were minced and boiled in 2 x SDS sample buffer. These samples were separated by SDS-PAGE and analysed immunologically. The expected cyclins and CDKs were detected with specific antibodies. A number of the expected cyclins and CDKs were found for p27^{wt} and unequivocally assigned to the respective protein bands of the pull down (figure 4.4) according to their mobility characteristics. In contrast and as expected the cyclin/CDK complexes were absent in the case of p27$^{\Delta CDK}$. This proves that this system can be used to identify new binding partners of p27. The remaining unknown

proteins were identified by mass spectroscopy and peptide sequencing (in cooperation with the working group of Prof. Dr. Friedrich Lottspeich at the "MPI für Biochemie") (fig. 4.3). The identified proteins can be divided into three groups.

4.3 Characterization of the interaction between Grb2 and p27

Grb2 (growth factor receptor-bound protein 2) was identified as a protein that interacts with p27. Grb2 is an adapter protein which has been ascribed functions in various signal transduction pathways. In this context it acts exclusively as a binding factor between proteins. Grb2 binds to phosphotyrosine residues via its central SH2 domain. In contrast the SH3 domains of the protein bind to proline-rich amino acid sequences of its interaction partner (figure 5.1). Hence the interaction Grb2-p27 is of particular interest because this is the first direct connection between signal transduction and the cell cycle that has been detected.

4.3.2 Characterization of the SH3 binding domain in p27

In order to characterize the binding of Grb2 to p27, four shortened versions of $p27^{wt}$ (lane 1), $p27^{wt\text{-}C\text{-}t}$ (aa 97-198, lane 5), $p27^{wt\text{-}N\text{-}t}$ (aa 1-96, lane 3), and the analogously shortened form of $p27^{\Delta CKI}$ (lane 2), $p27^{\Delta CKI\ N\text{-}t}$ (lane 4) were all produced by means of PCR in pET28a.

Interaction experiments with these recombinant protein fragments enabled the binding site of Grb2 in p27 to be localized in the N-terminal part of the protein (which also contains the CDK and cyclin binding sites). Moreover it was possible to confirm that the interaction between Grb2 and p27 occurs independently of intact binding sites for CDKs and cyclin (fig. 4.8 A). Computer-aided comparisons of various SH3 domain ligands with p27 yielded a proline-rich amino acid sequence directly adjacent to the CDK binding domain of p27.

This YYRPPRPPKGA motif in p27 corresponds to the XPPXPPX consensus sequence (X= charged aa, Sparks et al., 1996) which is necessary for binding to SH3

domains. Two essential prolines and a lysine in this domain were converted into glycine and alanine (P94G, P95G, K96A) by means of Quick Exchange PCR. This p27 variant ($p27^{\Delta SH3}$ in pET28a) was expressed recombinantly and tested for its interaction with Grb2. Grb2 can no longer bind $p27^{\Delta SH3}$ (fig. 4.8 B, lane 2). Thus the binding site for SH3 domain proteins in p27 was identified *in vitro* (fig. 4.8 B).

4.3.3 p27 interacts with the C-terminal SH3 domain of Grb2

p27 binds Grb2 via a proline-rich sequence. It may therefore be assumed that the interaction between Grb2 and p27 either occurs via the N-terminal or C-terminal SH3 domain of Grb2. In order to further characterize the binding of p27 in Grb2, GST fusions of the C-terminal and N-terminal SH3 domains and a GST fusion of the central SH2 domain of Grb2 (in pGEX-2T, in cooperation with Ottmar Jansen, Kiel) were expressed in *E. coli*. The binding of these domains to p27 was examined in interaction experiments. The GST fusions of the isolated Grb2 domains immobilized on glutathione Sepharose were incubated with recombinant p27 (ratio of the proteins to one another 1:1) and washed. The interacting proteins were separated by SDS-PAGE and blotted. A binding of p27 to the individual Grb2 domains was detected with a specific antibody against p27.

This experimental approach showed that p27 binds predominantly to the C-terminal SH3 domain of Grb2 (fig. 4.9). However, the N-terminal SH3 domain of Grb2 also interacts (6-7-fold weaker) with p27. In contrast the central SH2 domain of Grb2 does not interact with p27.

In order to investigate whether the proline-rich domain of p27 binds to further SH3 domains, 41 isolated SH3 domains of various signal transduction proteins were expressed in *E. coli* as GST fusions (pGEX-2T in cooperation with Ottmar Jansen, Kiel) and purified by glutathione Sepharose. The ability of these GST-SH3 fusion proteins to bind p27 was tested in an interaction experiment with purified, recombinant p27 (fig. 4.13). In addition to the known interaction of the C-terminal

SH3 domain of Grb2, only two other proteins, the isolated C-terminal SH3 domain of a second adapter protein (fig. 4.13 B, lane 16) and the isolated SH3 domain of the tyrosine kinase Lyn (fig. 4.13 A, lane 5) exhibited an interaction with p27 that was comparable or better than that of Grb2. In addition this relatively extensive study of various isolated SH3 domains showed that only the C-terminal SH3 domain of Grb2 can bind efficiently to p27 (fig. 4.13, lane 22 A and 18 B). The N-terminal SH3 domain (fig. 4.13, lane 21 A and lane 19 B) does not have a stronger affinity to p27 than most of the other examined SH3 domains.

4.4.1 Interaction of p27 with the tyrosine kinase Lyn

The isolated SH3 domain of the tyrosine kinase Lyn (p56) interacts with p27 *in vitro* (fig. 4.13). The aim was to confirm this interaction *in vivo* by immunoprecipitations. For this a 10 cm dish containing adherent HeLa cells was transfected with p27wt, $p27^{\Delta SH3}$ and $p27^{Y123/F}$ (Y74, Y88 and Y89/F, all constructs as HA fusion proteins in pCruz-HA). The cells were harvested 24 h after transfection, lysed and precipitated with a p27-specific antibody. After SDS-PAGE / immunoblot, co-precipitated Lyn was detected by a specific antibody to Lyn.

In addition to the detection of endogenous Lyn the same p27 constructs were cotransfected with Lyn-pApuro (isoform p56 of Lyn, obtained from Markus Warmuth, "Hämatologikum der GSF", Großhadern) and examined for an interaction of overexpressed Lyn with p27. In both cases a considerably increased binding of Lyn to the tyrosine deletion mutant of p27 ($p27^{Y123/F}$) was found (fig. 4.14, lanes 2 and 7).

In contrast only a very weak association with $p27^{wt}$ (fig. 4.14, lanes 1 and 6) was observed for endogenous or overexpressed Lyn. In addition the cell membranes of the co-transfection of $p27^{Y123/F}$ and Lyn obtained after ultrasonic disruption and centrifugation were isolated and dissolved in RIPA buffer. An immunoprecipitation

with anti-p27 antibody in the ten-fold diluted RIPA lysate showed a significant amount of membrane-bound p27 (fig. 4.14, lane 8).

4.5 Tyrosine phosphorylation of p27

The binding of tyrosine kinase to p27 *in vivo* would lead one to suspect that p27 could also be a direct substrate for phosphorylation by tyrosine kinases. Phosphorylation of the three tyrosines that are located exclusively in the inhibitory, N-terminal part of p27 (fig. 5.2) could be a mechanism for regulating the inhibitory activity of p27. The highly conserved inhibitory part of all three proteins of the Cip/Kip family have a conserved tyrosine (Y88 in p27). According to the crystal structure of p27 with the CDK2/cylcin A complex, tyrosine 88 of p27 is the amino acid which blocks the ATP binding pocket of the kinase subunit of the CDK complex (Russo *et al.*, 1996a). Since is has been shown that p27 competes with ATP for this binding site (Sheaff *et al.*, 1997), the phosphorylation of this tyrosine residue could change the inhibitory properties of p27.

4.5.1 The tyrosines of p27 and p21 are phosphorylated *in vivo*

$p27^{wt}$ binds *in vivo* to Lyn (see 4.4.1). The binding of a tyrosine kinase to p27 leads one to suspect that the tyrosines of p27 could be phosphorylated *in vivo*.

In order to set up the two-dimensional TLC technique for the special tyrosine phosphorylation of p27, p27 was phosphorylated *in vitro* in an experiment using [γ-^{32}P]ATP and Abl^{SH3+KD} (cf. 4.5.2 for details of this construct). In order to create the same conditions for the planned precipitation of endogenous p27, the radioactive phosphorylated p27 was incubated in MCF7 crude extract, boiled and subsequently precipitated by a specific p27 antibody. The precipitate was separated by SDS-PAGE and transferred to a PVDF membrane. The radioactive p27 was cut out of the membrane, hydrolysed and the phosphoamino acids were separated by two-dimensional TLC (fig. 4.16). In the case of *in vitro*-phosphorylated p27 only tyrosine phosphorylation was detected.

Hence adherent HeLa cells were cotransfected with p27 and a constitutively active form of Lyn and labelled with [^{32}P]-*ortho*-phosphate 24 h after the transfection. In addition the cells were incubated during the labelling with MG132 (100 µM) and LLnL (20 µM) to inhibit the proteasome. Phosphatases that may be active in vivo were inactivated by adding 100 µM Na ortho-vanadate, ocadeic acid (40 µM) to the medium and by a Na meta-vanadate treatment of the cells (by boiling activated Na ortho-vanadate at pH 10) during the last 30 min of the in vivo labelling. Overexpressed and stabilized p27 in this case showed a noticeable tyrosine phosphorylation. Serine at 72 % continued to be the amino acid that was modified most frequently, followed by tyrosine (16 %) and threonine (12 %). A tyrosine phosphorylation was also detected in the overexpression and stabilization of p21 in adherent HeLa cells. The cells transfected with p27 were labelled and stabilized together with the p27-transfected cells.

In the case of p21 the modification of serine at 74 % represents most of the total phosphorylation followed by 19 % for phosphotyrosine and 7 % for phosphothreonine. Further experiments will have to elucidate to what extent the tyrosine phosphorylation of p27 in this experiment is due to the overexpression of a constitutively active form of Lyn or to the stabilization of p27 by proteasomal inhibitors or the inactivation of endogenous cell phosphatases.

4.5.2 Specificity of the tyrosine phosphorylation

In addition to detecting the *in vivo* tyrosine phosphorylation of p27, the tyrosines of p27 were also phosphorylated *in vitro*. The aim was to use tyrosine-phosphorylated p27 to investigate *in vitro* whether there are any changes in the inhibitory properties of p27 or its binding to the CDK/cyclin complex. For this purpose the tyrosines of p27 were phosphorylated *in vitro* with the recombinantly-produced tyrosine kinases Abl and Src. Since c-Abl is too large to be expressed by bacteria and obtained in an actively folded form, c-Abl from mice (Abl in pRK5) was shortened by PCR such that the construct of the viral form obtained corresponded to the proto-oncogene v-

Abl (Abl^{KD}, aa 119 to 535). In addition a second form of Abl was produced which also contained the SH3 domain in addition to the SH2 and kinase domain (Abl^{KD+SH3}, aa 63-535). Both forms, Abl^{KD} and Abl^{KD+SH3} were cloned into pET28a as a hexahistidine fusion and expressed in *E. coli*. The soluble fractions of Abl^{KD} and Abl^{KD+SH3} were purified by affinity chromatography using a native nickel-chelate matrix (Abl is 1 -2 % soluble). A constitutively active form of Src (from chicken, also in pRK5,) was cloned completely into the expression vector pET28a. The protein Src is present in bacteria as an insoluble inclusion body pellet. This was purified to 80 % purity by an inclusion body purification procedure and washing and resuspending several times in 8 M urea. The Src kinase was subsequently denatured in 12 M urea at 50°C. Src was renatured by dilution according to the drop method.

The phosphorylatability the tyrosines of p27 in comparison to Sam68 (Santa Cruz Biotechnology) which is an established substrate for Abl and Src (Plattner et al., 1999) (fig. 4.19) was tested using these three recombinant kinases and a commercially available Src from Sf9 cells (Calbiochem-Novabiochem GmbH, Bad Soden).

Abl exhibits a far higher phosphorylation efficiency for p27 compared to the two Src variants. Also the ratio of the autophosphorylation of the kinases to the phosphorylation of the individual substrate proteins shows that p27 can be more efficiently modified by Abl. There was no difference between the two forms of the tyrosine kinases Abl, Abl^{KD} and Abl^{KD+SH3} with regard to the efficiency of p27 phosphorylation (fig. 4.19).

Hence Abl^{KD+SH3} was used in all subsequent *in vitro* tyrosine phosphorylations. In order to investigate the specificity of the tyrosine phosphorylation the three inhibitors of the Cip/Kip family, p21 (two tyrosines in the total protein), p27 (three tyrosines) and p57 (two tyrosines) were phosphorylated *in vitro* with Abl^{KD+SH3}. The GTP exchange factor for Ran, RanGAP-1 (three tyrosines, obtained from Dr. Frauke

Melchior, "MPI für Biochemie", Martinsried) and the BSA (21 tyrosines) added to the kinasation reaction for stabilization were used as controls (fig. 4.20). The tyrosines of all three inhibitor proteins but not those of the control proteins were phosphorylated. Similar results were obtained in a kinase reaction of p27 with Src^{rec}.

4.5.3 Identification of the phosphorylatable tyrosines in p27 and p21

p27 has a total of three tyrosines. These are all located in the N-terminal inhibitory part of the protein. In order to examine which of these three residues can be phosphorylated in vitro by Abl^{KD+SH3}, Src and Lyn, the tyrosines in p27 were substituted by the structurally similar phenylalanine. For this purpose the mutants $p27^{Y1/F}$ (Y74 to F), $p27^{Y2Y3/F}$ (Y88 and 89 to F), $p27^{Y123/F}$ (Y74, Y88 and Y89 to F), $p27^{Y2/F}$ (Y88 to F) and $p27^{Y3/F}$ (Y89 to F) were produced by Quick Exchange PCR and cloned into pET28a. All p27 variants were expressed recombinantly in *E. coli* and purified by a nickel-chelate affinity chromatography. After exactly matching the amounts of the p27 variants and $p27^{wt}$ by means of SDS-PAGE and Coomassie staining a tyrosine phosphorylation of the various p27 substrates was carried out with Abl^{KD+SH3} (fig. 4.21).

Tyrosine 74 is not recognized *in vitro* as a substrate, but tyrosines 88 or 89 are recognized as substrates. Tyrosine 89 exhibits a somewhat higher (1.3-fold) phosphorylation efficiency compared to tyrosine 88 in the unmodified form of p27. The measured incorporation of [γ-^{32}P]ATP shows that in each case only one of the two tyrosines is phosphorylated per molecule of p27. An initial phosphorylation of one of the two tyrosine residues probably sterically blocks the phosphorylation of the second tyrosine (fig. 4.21). The same result is obtained when Src is used instead of Abl^{KD+SH3}. A phosphorylation of the p28 amino acid exchange forms was carried out with the commercial (Upstate Biotechnology) tyrosine kinase Lyn. However, this did not lead to a utilizable result since the commercial kinase Lyn had a strong serine and threonine kinase activity. This contamination which is superimposed on the tyrosine

kinase activity does not allow any inferences to be made about a possible preference of Lyn for the three tyrosines in p27 (data not shown).

In order to test which tyrosine residues in p27 can be phosphorylation *in vitro*, $p21^{wt}$ and $p21^{Y1/F}$ (Y77 to F) and $p21^{Y2/F}$ (Y151 to F) were cloned into pET24d as C-terminal hexahistidine fusions. All three proteins were produced recombinantly, purified by a denaturing nickel-affinity matrix and renatured and concentrated by ion exchange chromatography. Kinase treatment of p21 was also carried out in this case with Abl^{KD+SH3}. This resulted in a modification of both tyrosine residues of p21 with comparable efficiency (data not shown).

4.5.4 Characterization of the binding of tyrosine-phosphorylated p27 to the CDK2/cyclin A complex

p27 is phosphorylated *in vitro* only on Y88 or Y89. Phosphorylation of these amino acids occurs in a region of p27 which is associated with the kinase part of CDK. This therefore raises the question whether a tyrosine phosphorylation of p27 only influences its binding to the kinase subunit of CKIs or whether the modification of the tyrosine residues of p27 also prevents its association with the cyclin subunit of the kinase.

This question was clarified using an analytical gel filtration of the ternary complex of tyrosine-phosphorylated p27 and CDK2/cyclin A. For this purpose an excess of free p27 was added to the recombinant CDK2/cyclin A complex purified from insect cells. The p27 was composed of a 1:1 mixture of unphosphorylated and *in vitro*-tyrosine-phosphorylated protein. CDK2/cyclin A and p27 were incubated and subsequently separated according to their respective Stoke's radius over a Superdex 200 HR 10/30 gel filtration column. The fractions obtained were evaluated by Coomassie staining after SDS-PAGE and by immunoblot analysis. A phosphotyrosine-specific antibody (4G10) was used to detect tyrosine-phosphorylated p27 (fig. 4.22 A). The specificity of the antibody was checked in an

immunoblot using unphosphorylated and *in vitro*-tyrosine-phosphorylated p27 (fig. 4.22 B). Tyrosine-phosphorylated p27 is located in the fractions of the gel filtration which are exclusively associated with CDK2/cyclin A. Consequently tyrosine-phosphorylated p27 binds the CDK complex. This binding may occur by means of an interaction with the cyclin part of the CDKs. However, modified p27 may also have altered binding properties with respect to the kinase subunit which is sufficient for binding but not for a complete inhibition of the kinase.

4.5.5 Change in the inhibitory activity of tyrosine-phosphorylated p27

p27 is phosphorylated *in vitro* on Y88 and Y89. According to X-ray structural data (Russo *et al.*, 1996a) these amino acids are the main sites of interaction of the inhibitor with the kinase subunit of the CKIs. It was therefore examined whether such a modification on tyrosine 88 or 89 results in a change in the inhibitory activity of p27. For this purpose recombinant $p27^{wt}$ and $p27^{Y123/F}$ with Abl^{KD+SH3} were tyrosine-phosphorylated *in vitro* by a relatively large amount (50 µg) of Abl^{KD+SH3}. In addition p27wt was treated similarly but without tyrosine kinase (mock control). All three preparations were boiled for 10 min at 100°C to inactivate the kinase, cooled for 10 min on ice and centrifuged. In order to separate unconsumed ATP, they were diafiltered four times against 150 mM NaCl, 50 mM Tris/HCl pH 7.5, 1 mM DTT and concentrated in a 2.5 ml Centrikon 10. The protein quantities of $p27^{wt}$, $p27^{Y123/F}$ and the mock control were matched by SDS-PAGE and Coomassie staining of the treated proteins. In addition a slight change in the migration properties of tyrosine-phosphorylated p27 compared to p27wt was observed in the SDS gel (see also fig. 4.26 B). The change in the migration property of p27 proved that p27 was completely phosphorylated.

In order to make inferences about the inhibitory potential of phosphorylated p27 compared to unphosphorylated p27, a histone H1 kinase assay was carried out. Identical initial amounts of the respective inhibitor were used for this in a titration series (fig. 4.23). Tyrosine-phosphorylated p27 exhibits a considerable shift in the

inhibition kinetics compared to mock-treated p27 and to $p27^{Y123/F}$ (fig. 4.23 C). This experiments shows that tyrosine-phosphorylated p27 has a ca. 40 % reduced inhibitory effect *in vitro* on the CDK2/cyclin A complex. In addition to histone H1 as a substrate of CDKs, the retinoblastoma protein (p110Rb) was tested which is the main target substrate of the cell cycle kinases *in vivo*. For this purpose commercially available pRb (QED Bioscience Inc., San Diego, California) was used in a kinase assay as a substrate of CDK2/cyclin A in order to determine the inhibitory activity of tyrosine-phosphorylated p27, of $p27^{wt}$ and of $p27^{Y123/F}$ were determined by titration (fig. 4.23 D). Also in this case an approximately 40 % attenuation of the inhibitory activity of tyrosine-phosphorylated p27 was found.

4.5.6 Tyrosine-phosphorylated p27 is phosphorylated to a greater extent on threonine 187

Tyrosine-phosphorylated p27 binds *in vitro* to the CDK2/cyclin A complex (see 4.5.4), but inhibits the CDK complex less efficiently. It is known from the literature that p27 is a substrate of CDK2/cyclin E and possibly also of CDK2/cyclin A. The phosphorylation of threonine 187 mediated by these kinases is the initial signal for the binding of p27 to the SCF^{SKP2} complex which is an E3 ligase of the ubiquitination machinery (Montagnoli *et al.*, 1999). Ubiquitinated p27 is subsequently proteasomally degraded (Pagano *et al.*, 1995). In order to examine whether an inhibitory-inactive tyrosine-phosphorylated p27 is a better substrate of the cell cycle kinases than unphosphorylated p27, a CDK2/cyclin A kinase assay was carried out using tyrosine-phosphorylated p27. For this purpose equal amounts of $p27^{wt}$ untreated by tyrosine kinase, tyrosine-phosphorylated p27 and $p27^{Y123/F}$ were phosphorylated identically with an excess of active CDK2/cyclin A complex.

Tyrosine-phosphorylated p27 is phosphorylated significantly better on threonine 187 by CDK2/cyclin A *in vitro* than $p27^{wt}$ and $p27^{Y123/F}$ (fig. 4.24). This 17.5-fold increase in the threonine 187-phosphorylation of modified p27 again shows the divergent inhibition properties compared to unphosphorylated p27 described in

section 4.5.5. The inactivation of p27 by a phosphorylation on the tyrosine residues may result in the phosphorylation of p27 on threonine 187 by the bound but active kinase (see 4.5.4). However, a phosphorylation of p27 on threonine 187 by CDK2/cyclin A could also take place transiently by another unbound kinase.

4.5.7 The tyrosine residues of p27 are not phosphorylated when it is present bound to the CDK2/cyclin A complex

The mechanism of tyrosine phosphorylation of p27 *in vitro* proves that the inhibitor is inactivated (fig. 4.23). In many cells most of the p27 is almost exclusively bound to CDKs (Hengst *et al.*, 1994; Steiner *et al.*, 1995). It was therefore investigated whether phosphorylation of tyrosine is also possible on a CDK/cyclin-bound p27. This phosphorylation would lead to an inactivation of complex-bound p27. In order to examine the phosphorylation of complex-bound p27, the ternary complex comprising recombinant p27 and CDK2/cyclin A from insect cells was purified by gel filtration at a stoichiometry of 1:1:1. A part of the complex purified in this manner was boiled for 10 min and centrifuged in order to release p27 again. These two samples, the CDK2/cyclin A-complexed p27 (fig. 4.25, lanes 1 and 2) and the free p27 (fig. 4.25, lane 3) were matched in amounts and phosphorylated with Abl^{KD+SH3}. Complex-bound p27 was phosphorylated as a control in order to determine the CDK2/cyclin A-mediated phosphorylation of p27 (fig. 4.25, lane 2). The CDK2/cyclin A-mediated phosphorylation of p27 was subtracted from the total phosphorylation of the tyrosine-phosphorylation. The free inhibitor is phosphorylated *in vitro* very much better (6.5-fold) by Abl^{KD+SH3} than the complexed inhibitor. p27 bound to CDK2/cyclin A may not be accessible to the tyrosine kinase. However, an increase in the phosphorylation by Abl was also detected for complex-bound p27 (fig. 4.25, lane 1). It remains to be proven whether this phosphorylation is due to a temporary dissociation of p27 from the CDK complex or whether p27 in the complex can be phosphorylated to a reduced extent. In contrast, the weaker phosphorylation of complexed p27 confirms the data from section 4.3.4. These demonstrate that Grb2 is not able to bind to a complex of p27 and CDK2/cyclin A. It remains to be

investigated whether a binding and phosphorylation of CDK-bound p27 by the tyrosine kinase Lyn is possible.

4.5.8 Mechanism of the binding and phosphorylation of p27 by the tyrosine kinase Lyn

The association of Lyn to p27 *in vivo* was more clearly demonstrated on a mutant of p27 in which all three phosphorylatable tyrosine residues were replaced by phenylalanine ($p27^{Y123/F}$, see 4.4.1). In contrast $p27^{wt}$ does not interact or interacts only weakly with Lyn. In contrast to $p27^{Y123/F}$ $p27^{wt}$ can be tyrosine-phosphorylated *in vivo*. As a result of this modification of p27, tyrosine-phosphorylated p27 may no longer be bound by the SH3 domain of the tyrosine kinase *in vivo*.

In order to examine whether a tyrosine phosphorylation of the inhibitor leads to a dissociation of the kinase from p27, an interaction assay was carried out with the GST fusion protein of the isolated SH3 domain of Lyn and a tyrosine-phosphorylated p27. The amounts of purified recombinant forms of p27 ($p27^{Y123/F}$, $p27^{\Delta SH3}$, $p27^{wt}$) and an *in vitro*-tyrosine-phosphorylated p27 were matched on the basis of SDS-PAGE and Coomassie staining (fig. 4.26 B). The same amounts of the inhibitors were incubated with an equal amount of the SH3 domain of Lyn immobilized on a glutathione Sepharose matrix. An interaction of p27 with Lyn-SH3 was detected immunologically after SDS-PAGE by means of a specific p27 antibody (fig. 4.26 A).

Tyrosine-phosphorylated p27 binds about 10-fold weaker to the SH3 domain of Lyn than $p27^{wt}$ or $p27^{Y123/F}$. Interestingly $p27^{\Delta SH3}$ binds more weakly to the SH3 domain of Lyn; however, a complete absence of an interaction as in the GST control of this experiment or in the case of the interaction of p27 and Grb2 (see fig. 4.9) is not observed. Hence the phosphorylation of tyrosine 88 or 89 in the direct vicinity of the SH3 binding domain in p27 leads *in vitro* to an increased dissociation of the kinase Lyn from the substrate p27.

4.5.9 Modification of p27 on tyrosine 88 or 89 inactivates and destabilizes p27 *in vivo*

The tyrosine phosphorylation of p27 weakens the activity of the inhibitor *in vitro* (see 4.5.5). In addition an initial tyrosine modification of p27 facilitates a subsequent phosphorylation of p27 on threonine 187 (section 4.5.6). This phosphorylation is the specific signal for the recognition of p27 by the SCF^{SKP2}-E3 ligase complex and the associated degradation of the molecule *in vivo* (Krek, 1998; Montagnoli *et al.*, 1999).

In order to examine whether the tyrosine phosphorylation of p27 leads to an inactivation and destabilization of the inhibitor *in vivo*, an *in vivo* kinase assay and a half-life determination (pulse chase labelling) of p27 was carried out. For the *in vivo* kinase assay a 10 cm dish of adherent HeLa cells was transfected in each case with a constitutively active form of Lyn (pApuro-Lyn, obtained from Markus Warmuth, "Hämatologikum der GSF", Großhadern) and with HA-$p27^{wt}$ and HA-$p27^{Y123}$ (both in pCruz-HA). The cells were lysed 24 h after the transfection and the cyclin A/CDK complexes were immunoprecipitated from identical amounts of total protein by means of an anti-cyclin A antibody. An immobilized histone H1 kinase assay was carried out using the precipitated kinase and the co-precipitated overexpressed inhibitors. After evaluation of the radioactive signals by means of a Phosphoimager (Fujifilm BAS-2500 Phosphoimager, Fujifilm) the amounts of precipitated CDK2 and p27 were analysed by specific antibodies and the signals from the antibody detection were determined by densitometry (ImageMaster 1D Elite program, Amersham).

Quantification of the kinase activity taking into account the quantification of the signals obtained from the antibody detection of CDK2 and p27 shows that $p27^{wt}$ exhibits an approximately 50 % reduction of its inhibitor activity *in vivo* compared to $p27^{Y123/F}$. This change in activity is in agreement with the change in activity of tyrosine-phosphorylated p27 that was determined *in vitro* (see 4.5.5). This suggests that p27 can also be inactivated *in vivo* by a tyrosine phosphorylation. However, only

a small amount of CDK2 was detected in the precipitation of the inhibitors (fig. 4.27 anti-HA, lanes 1 and 2). Hence it must be assumed that p27 was in excess over the kinase in the subsequent kinasation reaction. However, a longer exposition of the kinase reaction also showed an increased inhibition in the case of the tyrosine substitution mutant of p27.

It mains to be clarified to what extent the increased kinase activity in the precipitation with antibodies against cyclin A is due to a free kinase. On the other hand, in order to check whether initial tyrosine phosphorylation of p27 contributes to a destabilization of the inhibitor *in vivo,* a metabolic labelling of p27 with [^{35}S] methionine and [^{35}S] cysteine (pulse chase) was carried out to determine the half-life. The activities obtained after 0, 1, 2 and 5 h for the various forms of p27 were quantified by a Phosphoimager and adjusted according to the amount of protein used and the total incorporation of [^{35}S]. p27$^{\Delta SH3}$ exhibited a considerable increase in stability (4 h) compared to p27^{wt} (2.5 h). p27$^{Y123/F}$ also had a somewhat longer half-life of 3.5 h. This result suggests that the SH3 domain binding region in p27 as well as the phosphorylation of the tyrosines affects the stability of p27 *in vivo*. This indicates that a tyrosine kinase probably interacts *in vivo* with the inhibitor via the proline-rich region of p27. p27 is subsequently phosphorylated on tyrosine and inactivated. Tyrosine-modified p27 is in turn degraded to an increased extent.

4.5.10 Characterization of various mutated forms of p27

In this experiment various mutated forms of p27 were investigated (p27$^{Y1/F}$, p27$^{Y23/F}$, p27$^{Y123/F}$ and p27$^{\Delta SH3}$). An inhibitor kinase assay was carried out with histone H1 as a substrate of the cell cycle kinases in order to ensure that the function of these forms was comparable to the activity of p27^{wt} (fig. 4.29). The examined forms of p27 have a comparable inhibitory activity to p27^{wt}. The increased inhibition of p27$^{\Delta SH3}$ is due to a somewhat higher amount of inhibitor that was used.

In addition to the activity of the mutated kinase inhibitors, the location of $p27^{Y123/F}$ and $p27^{\Delta SH3}$ was examined in relation to the exclusively nuclear location of $p27^{wt}$. For this the HA fusion constructs (in pCruz-HA) of $p27^{Y123/F}$, $p27^{\Delta SH3}$ and $p27^{wt}$ were transfected into adherent HeLa cells and examined by immunocytochemistry 24 h after the transfection. Also in this case the mutated forms of p27 exhibited the same exclusively nuclear location as the unmutated form of p27.

4.5.11. p27 is phosphorylated on tyrosine residue 88 *in vivo*.

Antibodies specific for a peptide containing phosphorylated tyrosine 88 were generated in rabbits (figure 4.31). The affinity purified antiserum specifically reacts with overexpressed HA-tagged p27 isolated from cells that coexpress either the kinase Bcr-Abl or Lyn. Phosphatase treatment abolished the signal without reducing the total amount of p27, demonstrating specificity of the antiserum (figure 4.31). Subsequently, p27 was transfected in the presence or absence of Lyn or BcrAbl kinases. Similar amounts of immunoprecipitated p27 were loaded and separated by SDS-PAGE. Even in the absence of overexpressed kinase a signal is obtained with the phosphoform specific antibody, demonstrating phosphorylation on tyrosine 88 in cells transfected with only p27. The signal is strongly enhanced by overexpressing Lyn or BcrAbl, demonstrating that these kinases can phosphorylate p27. A mutant p27 protein where tyrosines 88 and 89 were exchanged to phenylalanine lacks any cross reactivity with the anitserum (figure 4.32). These data confirm that the tyrosine residue 88 of p27 can become phosphorylated *in vivo* and that overexpression of tyrosine kinases like Lyn and abl strongly enhance the phosphorylation on tyrosine 88.

5 Discussion

The CDK inhibitor $p27^{Kip1}$ plays a key role in controlling CDK kinase activity during

the G1 / S transition. Factors which regulate level or activity of the inhibitor p27 are thus of fundamental importance for an understanding of cell cycle control. We report here the identification of p27 as a substrate for tyrosine phosphorylation. Tyrosine kinases preferably phosphorylate p27 at tyrosine 88. This phosphorylation impairs the growth inhibitory function of the CDK inhibitor protein. Generation of phospho-specific antibodies against the modified protein allows a distinction to be made between the inhibitory and modified forms of p27, thus significantly improving its value as a prognostic or monitoring marker.

There are four potential mechanisms by which p27, p21 or p57 may become substrates of tyrosine kinases. I. Some kinases like Lyn may directly interact with the inhibitor p27 and then phosphorylate the bound protein. II. Even though Bcr-Abl does not stably binds to p27 and purified Abl kinase phosphorylates the inhibitor in vitro, an adaptor like Grb2 may facilitate binding to p27 *in vivo*. Grb2 and other adaptors may recruit p27 to other kinases including receptor tyrosine kinases. III. The kinase may not stably bind to p21, p27 or p57 and a very transient interaction may be sufficient for phosphorylation. IV. Proteins within inhibitor containing complexes like the pRb complex might recruit tyrosine kinases to p27. The tyrosine kinase Abl binds to pRb (Welch and Wang, 1993). As a result of the interaction of the CDK complexes with pRb, the CDK inhibitor associated with the CDK complexes could come into contact with the tyrosine kinase Abl and be modified. This pathway would also be independent of SH3 domains.

The p27/Grb2 interaction

Grb2 (growth factor receptor-bound protein 2) is an adapter protein of 217 amino acids in size which has a central SH2 domain flanked by two SH3 domains. As an adapter protein Grb2 binds to tyrosine-phosphorylated proteins via its SH2 domain like the receptors of growth factors (EGFRs and PDGRFs: Lowenstein *et al.*, 1992;

van der Geer and Hunter, 1993). In addition Grb2 interacts via its two SH3 domains with short proline-rich regions in protein ligands (Kohda *et al.*, 1994). In this manner Grb2 interacts with the carboxy-terminal region of the guanine nucleotide exchange factor Sos (son of sevenless) which is an activator of Ras (Li et al., 1993; Rozakis-Adcock *et al.*, 1993). Thus Grb2 links receptor mediated tyrosine kinase activation to the Ras signal transduction cascade (Baltensperger *et al.*, 1993; Gale *et al.*, 1993). Here, Grb2 was identified as a protein that can bind to the CDK-inhibitor p27.

In further investigation of the interaction of p27 with Grb2 it was shown that Grb2 binds to p27 in a proline-rich region next to the CDK binding domain of the inhibitor. This binding site was confined to p27; p21 and p57 do not bind to Grb2. Two prolines (P94, P95) and a lysine (K96) are essential for the interaction with Grb2. It was shown that Grb2 binds to p27 via its C-terminal SH3 domain.

Recombinant p27 cannot bind unmodified Grb2 *in vitro*, when p27 is present as a CDK/cyclin-bound protein as shown in this study. The CDK/cyclin complex also displaces Grb2 from its binding to p27. Consequently recombinant Grb2 is not able to modulate the activity of the inhibitor *in vitro*. Thus an interaction p27-Grb2 appears only to be restricted to p27 not bound to CDKs or after modification of either p27 or Grb2. Since p27 was present in an unmodified form in the pull down experiment, it can be assumed that p27, when present at elevated concentrations, does not require any post-translational modification for interaction with Grb2. One could conceive that Grb2 is specifically modified as a result of stimulation of the signal transduction cascade which allows it to then bind to p27. Thus a phosphorylation of Grb2 in its C-terminal SH3 domain has been described which influences its interaction with the protein ligand of the adapter. The phosphorylation of Grb2 is initiated by the binding of the tyrosine kinase Abl to the SH2 domain of Grb2 (Li *et al.*, 2001). The modified Grb2 could thus subsequently bind to factors of the membrane or cytoplasm and be retained there. An interaction p27-Grb2 could for

example only take place with unbound cytoplasmic p27 after prior stimulation-dependent activation of Grb2.

The regulated import of p27 into the nucleus may be affected by a binding to Grb2 or Lyn. Müller *et al.*, (2000) showed that NPAP60, which is a nuclear pore associated protein, binds to p27. This interaction influences the location of p27. The binding of NPAP60 takes place in the 3-10-helix motif of the inhibitor at involves arginine 90. The binding of Grb2 in this region of p27 (binding domain amino acids 90-96) could lead to a competition of the nuclear pore protein with the adapter molecule Grb2. A blockade of the NPAP40-mediated import of p27 by Grb2 could thus also have effects on the location and activity of the inhibitor.

The interaction of Grb2 and p27 led to the speculation that other SH3 binding domains might interact wth p27. Therefore we screened isolated SH3 domains for their ability to bind to p27. Using this approach, we identified the tyrosine kinase Lyn. Binding of the tyrosine kinase Lyn suggested that p27 can be phosphorylated directly on tyrosine residues.

Lyn is one of the non-receptor tyrosine kinases of the Src family and, like Src itself, has an SH3, SH2 and kinase domain in addition to a so-called C-terminal unique region. Lyn is mainly expressed in haematopoetic tissues (summarized in Hibbs and Dunn, 1997). The kinase is activated in the late G1 phase (Mou and Linnekin, 1999). A deregulated activity of the tyrosine kinase is one of the main causes of chronic myeloid leukaemia (Harder *et al.*, 2001; Roginskaya *et al.*, 1999).

In a first step the interaction between Lyn and p27 was confirmed *in vivo*. The increased binding of the kinase to a form of p27 that could no longer be

phosphorylated was notable in the immunoprecipitation carried out in this connection. This indicated a phosphorylation of p27 by Lyn. Binding of Lyn to p27 requires therefore no additional adapter protein or complex formation with the CDK complex. Lyn has been described to interact with CDK2 after stimulation with 1- β-D-arabinofuranosyl cytosine (Mou and Linnekin, 1999; Yuan *et al.*, 1996). In U-937 cells this interaction leads to a phosphorylation of CDK2 at tyrosine 15 and to an inactivation of the kinase. The interaction between Lyn and CDK2 may be mediated by the p27.

The immunoprecipitation experiments showed also an interaction of p27 with Lyn on the plasma membrane. It was possible to isolate p27 from a purified fraction of the plasma membrane. This membrane-associated p27 interacted with Lyn. Yaroslavskiy *et al.*, (2001) showed that p27 is also found on the plasma membrane despite its predominantly nuclear location. p27 was located in detergent-insoluble microdomains of the lymphocyte membrane. It is conceivable that p27 is brought to the plasma membrane by adapters like Grb2 or by the tyrosine kinase Lyn. All three proteins are able to interact with the receptors of signal transduction that are stably integrated into the plasma membrane (Hibbs and Dunn, 1997; Lowenstein *et al.*, 1992; Skolnik *et al.*, 1993). However, it remains unclear which precise function p27 has at the membrane. Membrane associated p27 might also have a function in the organisation and regulation of the cytoskeleton. Sun et al., (2001) discuss a role of p27 in the migration of smooth muscle cells

5.2 The tyrosine phosphorylation of p27

We observed that p27 can be phosphorylated on tyrosine residues by a number of tyrosine kinases. The position of phosphorylatable tyrosines in p27 is important for an understanding of the function of the interaction between the signal transduction proteins and p27 . Three tyrosines in p27 are located in the CDK-inhibitory domain

of p27. The two directly adjacent tyrosines 88 and 89 are of particular interest. According to X-ray structural data of the complex of p27 with CDK2/cyclin A (Russo *et al.*, 1996a) tyrosine 88 in particular is the amino acid which is most probably involved in the interaction between the inhibitor and kinase subunit and kinase inhibition. Tyrosine residue 88 occupies the purine binding pocket of the kinase subunit, preventing binding of ATP. The competition of free p27 with ATP prevents the phosphorylation of substrate proteins by CDK (Sheaff *et al.*, 1997). A modification of p27 at tyrosine 88 should lead to a complete change in the interaction between CDK and the inhibitor. A modulation in the activity of the inhibitor has possibly effects on the activity of the CDKs. In addition, CDK2 bound p27 might now become a substrate of this kinase since it may not longer block the ATP-binding pocket of the kinase.

It was possible to detect a direct tyrosine phosphorylation of p27 *in vivo* by radioactive metabolic labelling of transfected p27 and subsequent separation of the phosphoamino acids in a thin layer chromatogram in HeLa cells.A 16 % tyrosine phosphorylation of p27 was obtained by treating HeLa cells with phosphatase and proteasome inhibitors and co-transfecting these cells with p27 and Lyn. In this case the serine phosphorylation was 74 % and the threonine phosphorylation was 12 % of the total phosphorylation. It was shown that p27 can only be modified on tyrosine 88 and 89 by the recombinant tyrosine kinase Src or Bcr-Abl or the truncated kinase domain of the tyrosine kinase Abl. Consistent with the *in vitro* experiments, generation of phospho-specific antibodies demonstrate that tyrosine residue 88 of p27 is modified *in vivo* by either Src, Bcr-Abl or Lyn kinase.

Tyrosine phosphorylation of recombinant p27 in a kinase assay with histone H1 or pRb leads *in vitro* to a reduction of the inhibitory activity of p27 by about 40 %. This suggests that p27 is inactivated after tyrosine phosphorylation. Reduced CDK

inhibitory activity could transform the inhibitor into an activator of cyclin D/CDK4,6 kinases. This modification might explain the controversial observations on the activity of p27-bound CDK4/D-typ cyclin complexes. In early G1 phase tyrosine phosphorylated p27 might become growth promoting by stimulating assembly of cyclin D/CDK4,6 kinase complexes.Tyrosine phosphorylated p27 further becomes a better substrate for CDK2-dependent phosphorylation. It was previously shown that phosphorylation of p27 on threonine 187 leads to ubiquitination of the inhibitor through the SCF-Skp2 E3-ubiquitin-ligase. Polyubiquitinated p27 is subsequently degraded by the 26S proteasome (Montagnoli *et al.*, 1999; Pagano *et al.*, 1995).

Both observations indicate that the phosphorylation state of p27 may be essential for determination its prognostic value. Whereas unmodified p27 may primarily inhibit cell proliferation, the opposite may be true for tyrosine phosphorylated p27: Early in G1 this form of the protein may even act as an activator by stimulating assembly of CDK4,6 with D-type cyclins. Later in G1 the same modification may initiate the proteolysis of the inhibitor and make it unstable prior to its intrinsic destabilisation after CDK2 activation.

A CDK inhibitory domain lacking tyrosines in position homologue to 88 and 89 of p27 should be resistant to modifications by tyrosine kinases and therefore be of specific therapeutic value. This form should be stabilised compared to the wild-type allele and therefore more efficient in inhibiting cell proliferation.

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Claims

1. A polypeptide comprising the amino acid sequence SEQ ID NO: 2, 4 or 6 whereby in the polypeptide comprising the amino acid sequence SEQ ID NO: 2, the tyrosine residue at position 88 and/ or the tyrosine residue at position 89 in SEQ ID NO: 2 is/ are phosphorylated, or
whereby in the polypeptide comprising the amino acid sequence SEQ ID NO: 4, the tyrosine residue at position 77 in SEQ ID NO 4 is phosphorylated, or
whereby in the polypeptide comprising the amino acid sequence SEQ ID NO: 6, the tyrosine residue at position 91 in SEQ ID NO 6 is phosphorylated.

2. A polypeptide according to claim 1 comprising the amino acid sequence SEQ ID NO: 2,
whereby the tyrosine residues at position 88 and/ or the tyrosine residue at position 89 in SEQ ID NO: 2 are phosphorylated and whereby the serine residue at position 10 and/or 12 and/or the threonine residue at position 157 and/or the threonine residue at position 187 in SEQ ID NO: 2 are phosphorylated.

3. A peptide fragment with a minimum length of 6 amino acids of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, 4 or 6,
whereby in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, the tyrosine residue at position 88 and/ or the tyrosine residue at position 89 in SEQ ID NO: 2 is/ are phosphorylated, or
whereby in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 4, the tyrosine residue at position 77 in SEQ ID NO 4 is phosphorylated, or
whereby in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 6, the tyrosine residue at position 91 in SEQ ID NO 6 is phosphorylated.

4. A peptide fragment according to claim 3 with a minimum length of 10 amino acids, preferably with a minimum length of 15 amino acids.

5. A polypeptide
comprising the amino acid sequence SEQ ID NO: 2, 4 or 6 or
comprising the amino acid residues 1 to 95 of SEQ ID NO: 2 or comprising the amino acid residues 1 to 85 of SEQ ID NO: 4 or comprising the amino acid residues 1 to 100 of SEQ ID NO: 6 or
comprising the amino acid residues 50 to 95 of SEQ ID NO: 2 or comprising the amino acid residues 38 to 85 of SEQ ID NO: 4 or comprising the amino acid residues 50 to 100 of SEQ ID NO: 6
characterized in that
in the polypeptide derived from the polypeptide with the amino acid sequence SEQ ID NO: 2, the amino acid residue at position 88 and/or position 89 in SEQ ID NO: 2, or
in the polypeptide derived from the polypeptide with the amino acid sequence SEQ ID NO: 4, the amino acid residue at position 77 in SEQ ID NO: 4 or
in the polypeptide derived from the polypeptide with the amino acid sequence SEQ ID NO: 6, the amino acid residue at position 91 in SEQ ID NO: 6
is a non-phosphorylable amino acid residue, preferably a phenylalanine residue.

6. A peptide fragment with a minimum length of 6 amino acids of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, 4 or 6
characterized in that
in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 2, the peptide fragment comprises at least one of the flanking amino acid residues of the amino acid residue at position 88 or 89 in SEQ ID NO: 2 and the residue at position 88 and/ or the residue at position 89 in SEQ ID NO: 2 is a non-phosphorylable amino acid residue, preferably a phenylalanine residue, or

in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 4, the peptide fragment comprises at least one of the flanking amino acid residues of the amino acid residue at position 77 in SEQ ID NO: 4 and the residue at position 77 in SEQ ID NO: 4 is a non-phosphorylable amino acid residue, preferably a phenylalanine residue, or
in the peptide fragment of a polypeptide comprising the amino acid sequence SEQ ID NO: 6, the peptide fragment comprises at least one of the flanking amino acid residues of the amino acid residue at position 91 in SEQ ID NO: 6 and the residue at position 91 in SEQ ID NO: 6 is a non-phosphorylable amino acid residue, preferably a phenylalanine residue.

7. A nucleic acid molecule encoding a polypeptide according to claim 5 or a peptide fragment according to claim 6.

8. An isolated antibody which specifically binds to a polypeptide according to any of the claims 1 to 2 or to a peptide fragment according to the claims 3 or 4 and which has less than 10% cross reactivity with the non-phosphorylated polypeptide or non-phosphorylated peptide fragment.

9. The antibody of claim 7, wherein the antibody is a monoclonal antibody.

10. The antibody of claim 7, wherein the antibody is a polyclonal antibody.

11. A polypeptide according to claim 5, a peptide fragment according to claim 6, a nucleic acid molecule according to claim 7 or an antibody according to any of the claims 8 to 10 for use in medicine.

12. A pharmaceutical composition comprising a polypeptide according to claim 5, a peptide fragment according to claim 6, a nucleic acid molecule according to claim 7 or an antibody according to any of the claims 8 to 10.

12. Use of a polypeptide according to claim 5, a peptide fragment according to claim 6, a nucleic acid molecule according to claim 7 or an antibody

according to any of the claims 8 to 10 for the preparation of a pharmaceutical composition for the treatment of hyperproliferative disease, preferably cancer.

13. A method for the determination of the amount or presence of a polypeptide according to any of the claims 1 or 2 or a peptide fragment according to any of the claims 3 or 4 in a sample comprising the steps of
a) providing a sample suspected to contain the polypeptide or the peptide fragment,
b) incubating the sample in the presence of an antibody according to any of the claims 8 to 10, and
c) determining the binding product between the polypeptide and the antibody thereby concluding that the polypeptide or peptide fragment is present or thereby deriving the amount of the polypeptide or the peptide fragment.

14. A kit for the detection or determination of the amount of the polypeptide according to any of the claims 1 to 2 or the peptide fragment according to any of the claims 3 or 4 in a biological sample which comprises:
(a) an antibody according to any of the claims 8 to 10 and
(b) a label for qualitatively or quantitatively detecting an immunoconjugate of the antibody and the polypeptide or the peptide fragment.

15. A method of determining whether or not a human cancer cell containing patient sample has potential for tumor progression, the method comprising comparing:

a) the level of expression a polypeptide according to any of the claims 1 to 2 or a peptide fragment according to claim 3 or 4 in the patient sample, and
b) the normal level of expression the polypeptide or the peptide fragment in a sample from a control subject not afflicted with cancer,

and a significant difference between the level of expression of the polypeptide or the peptide fragment in the patient sample and the normal level of the polypeptide or the peptide fragment in the sample from a control subject not

afflicted with cancer is an indication that the patient sample has potential for tumor progression.

16. The method according to claim 15, wherein the sample is a tissue sample, blood or blood derived cells, primary cell cultures from patients, stool, lymph or a tissue-associated fluid or urine.

17. The method of claim 15 to 16, wherein the presence of said polypeptide or peptide fragment is detected using a reagent which specifically binds with said polypeptide or peptide fragment.

18. The method of claim 11, wherein the reagent is selected from the group consisting of an antibody, an antibody derivative, and an antibody fragment.

19. A method of selecting a composition for inhibiting the progression of cancer in a patient, the method comprising:

a) providing a sample comprising cancer cells from the patient;
b) separately exposing aliquots of the sample in the presence of a plurality of test compositions;
c) comparing expression of the polypeptide according to any of the claims 1 to 2 or a peptide fragment according to any of the claims 3 or 4 in each of the aliquots; and
d) selecting one of the test compositions which alters the level of expression of the polypeptide in the aliquot containing that test composition, relative to other test compositions.

20. A method of deriving a candidate agent, said method comprising:

(a) contacting a sample containing cancer cells, with said candidate agent;
(b) determining the level of expression of the polypeptide according to any of the claims 1 to 2 or a peptide fragment according to any of the claims 3 or 4 in the sample contacted with the candidate agent and determining

the level of expression of the polypeptide in a sample not contacted with the candidate agent;

(c) observing the effect of the candidate agent by comparing the level of expression of the polypeptide or the peptide fragment in the sample contacted with the candidate agent and the level of the polypeptide or the peptide fragment in the sample not contacted with the candidate agent,

(d) deriving said agent from said observed effect,

wherein an at least 1.5 fold difference or a less than 0.75 fold difference between the level of expression of the polypeptide or the peptide fragment in the sample contacted with the candidate agent and the level of expression of the polypeptide or the peptide fragment in the sample not contacted with the candidate agent is an indication of an effect of the candidate agent.

21. The method according to Claim 20, wherein said candidate agent is a candidate inhibitory agent.

22. The method according to Claim 20, wherein said candidate agent is a candidate enhancing agent.

23. Use of a polypeptide according to any of the claims 1 to 2 or 5, preferably according to claim 1 or 2, or of a peptide fragments according to any of the claims 3 to 4 or 6, preferably according to claims 3 to 4, for the determination of the potential of a human cancer cell for tumor progression.

24. Use of a nucleic acid molecule according to claim 7 for the expression of a polypeptide according to claims 5 or a peptide fragment according to claim 7.

25. Use of a polypeptide according to any of the claims 1 to 2 or 5, preferably according to the claim 1 or 2, or of a peptide fragment according to any of the claims 3 to 4 or 6, preferably according to the claims 3 to 4, as an immunogen to generate or produce antibodies, in particular monoclonal antibodies.

Abstract

The present invention is directed to tyrosine phosphorylated forms of p27Kip1, fragments of these forms and antibodies thereto. The invention is further related to non-phosphorylable forms of p27Kip1 or fragments thereof. Further embodiments are diagnostic or therapeutic uses of the disclosed compounds, in particular uses in diagnostics and therapy of hyperproliferative diseases.

Figure 1

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                          10         20         30         40
                          |          |          |          |
p27 protein    1   MSNVRVSNGSPSLERMDARQAEHP--KPSACRNLFGPVDHEELTRD---L
p57 protein    1   MSDASLRS-TSTMERLVARGTFPVLVRTSACRSLFGPVDHEELSRE---L
p21 protein    1   MS-----------EPAGDVRQNPC--GSKACRRLFGPVDSEQLRRDCDAL

                       50         60         70         80         90
                       |          |          |          |          |
p27 protein   46   EKHCRDMEEASQRKWNFDFQNHKPLEG--KYEWQEVEKGSLPEFYYRPPR
p57 protein   47   QARLAELNAEDQNRWDYDFQQDMPLRGPGRLQWTEVDSDSVPAFY--RET
p21 protein   38   MAGC--IQEARER-WNFDFVTETPLEG--DFAWERVRGLGLPKLYL-PTG

                      100                                 110        120
                       |                                  |          |
p27 protein   94   PPKGACKV-----------------PAQES----QDVSGSRPAAPLIGA
p57 protein   95   VQVGRCRLLLAPRPVAVAVAVSPPLEPAAESLDGLEEAPEQLPSVPVPAP
p21 protein   82   PRRG-------------------------R----DELGGGRRP------

                     130        140        150
                      |          |          |
p27 protein  122  PANSEDTHLVDPKTDPSDSQTGLAEQCAGI-------------------
p57 protein  145  ASTPPPVPVLAPAPAPAPAPVAAPVAAPVAVAVLAPAPAPAPAPAPAPAP
p21 protein   96  ---GTSPALLQGTAE--EDHVDLSLSCTLV-------------------

                                 160        170
                                  |          |
p27 protein  152  -----------RKRPATDDSSTQNKRANRTEENVSDG-------------
p57 protein  195  VAAPAPAPAPAPAPAPAPAPDAAPQESAEQGANQGQRGQEPLADQLHS
p21 protein  121  -----------P-RSGEQAEGSPGGPGDSQGRKRRQT-------------

                                       180        190
                                        |          |
p27 protein  178  ------------------------SPNAGSVEQTPKKPGL----------
p57 protein  245  GISGRPAAGTAAASANGAAIKKLSGPLISDFFAKRKRSAPEKSSGDVPAP
p21 protein  146  ------------------------S--MTDFYHSKRRLIF----------


p27 protein  194  -----------------RRRQT
p57 protein  295  CPSPSAAPGVGSVEQTPRKRLR
p21 protein  160  -----------------SKRKP
```

Fig. 4.1

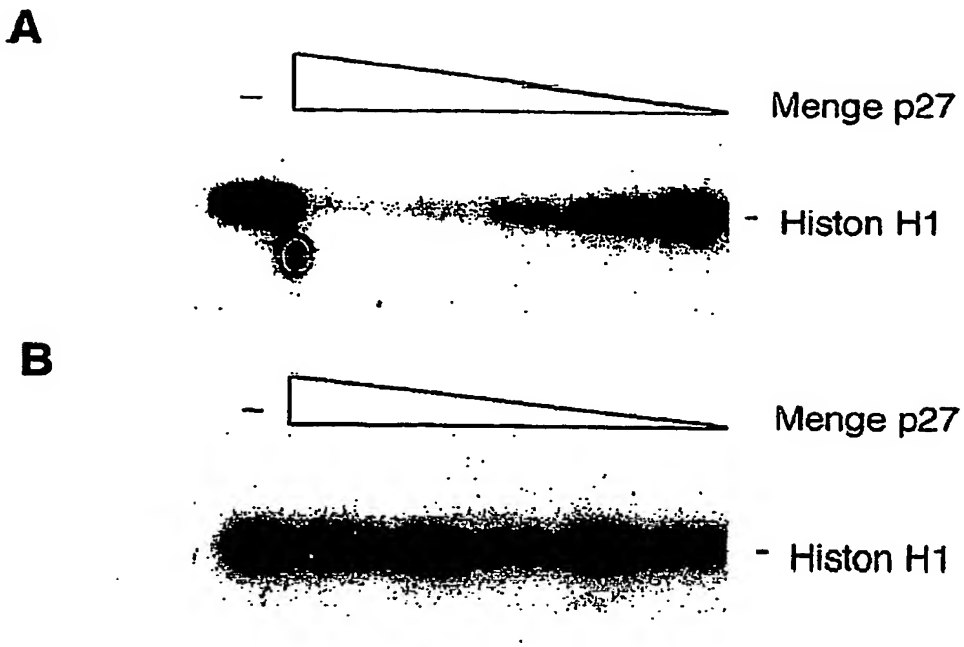


Fig. 4.2:

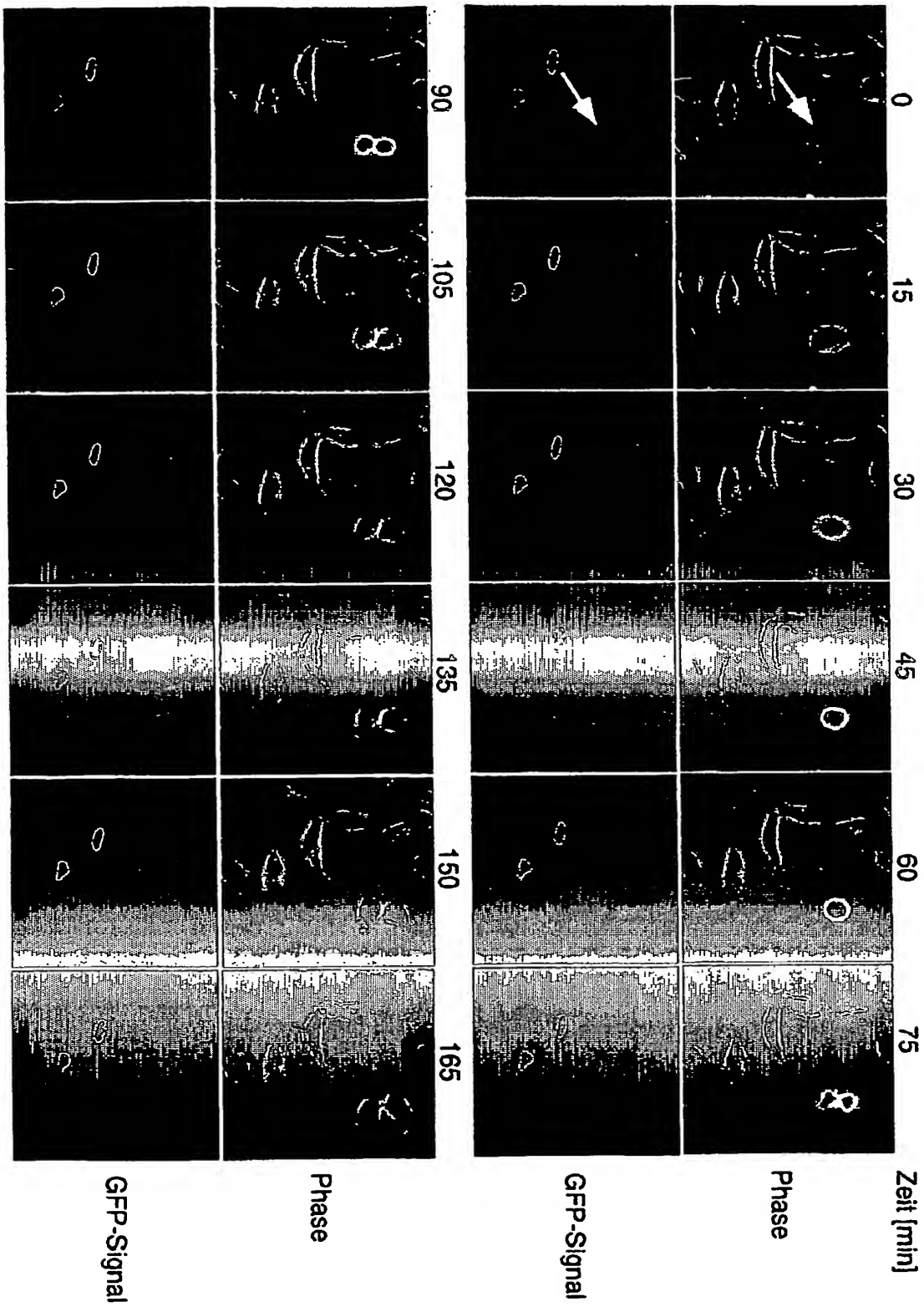

0
15
30
45
60
75
Zeit [min]
Phase
GFP-Signal
90
105
120
135
150
165
Phase
GFP-Signal

Fig. 4.3.:

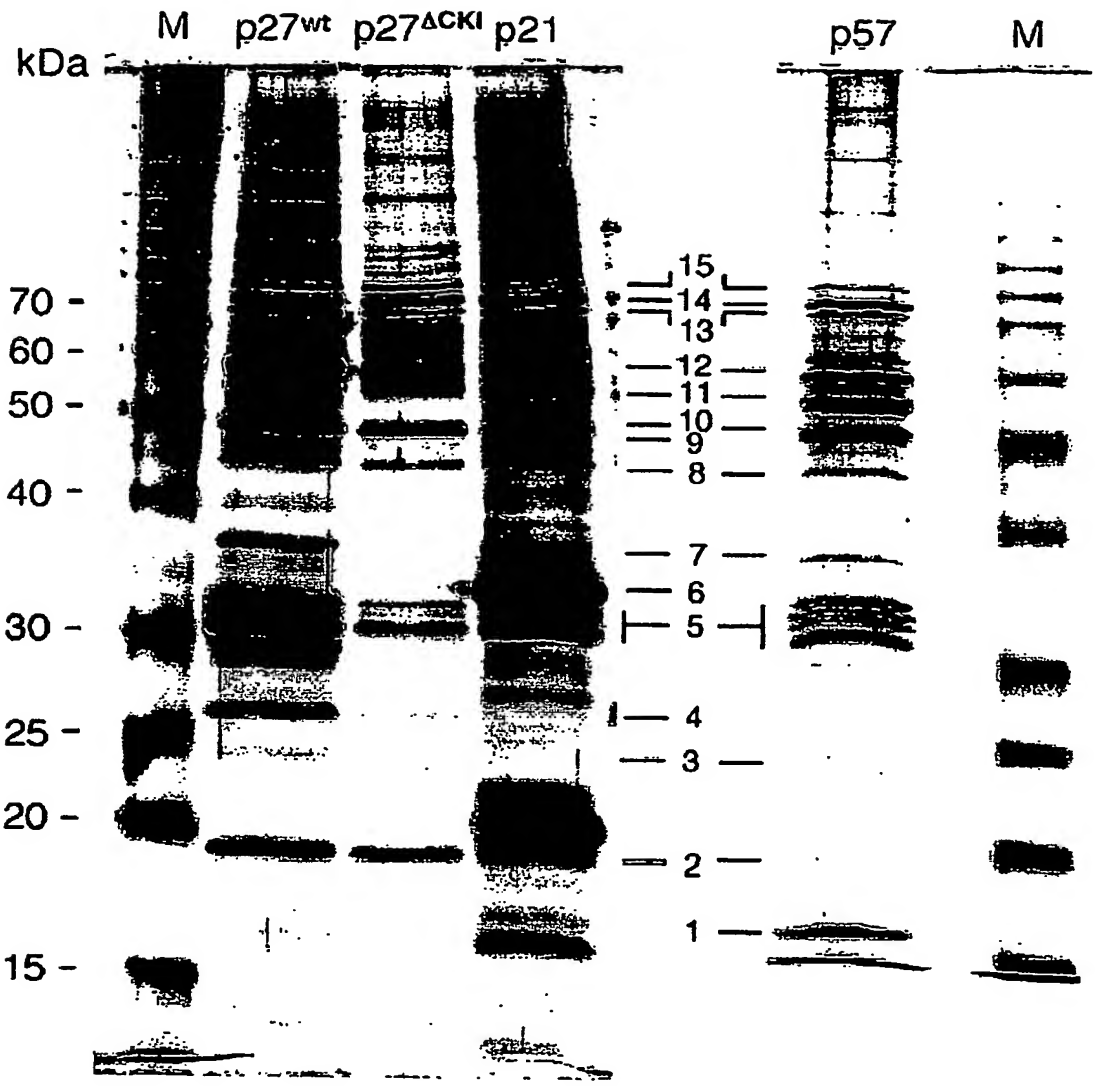

M
p27wt
p27ΔCKI
p21
p57
M
kDa
70 -
60 -
50 -
40 -
30 -
25 -
20 -
15 -
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1

Fig. 4.4:

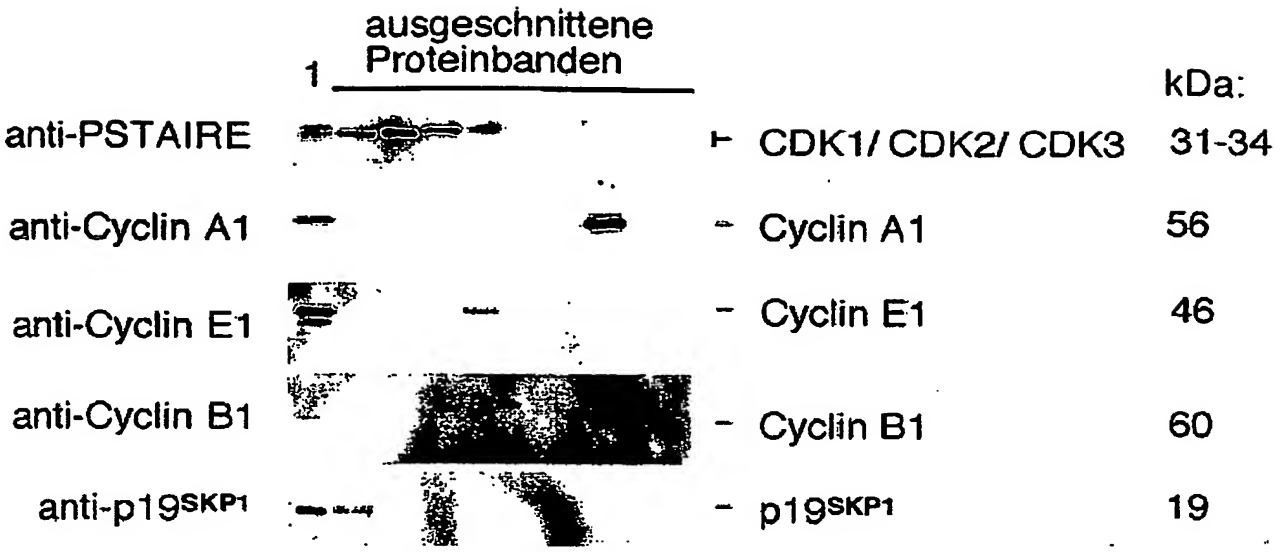
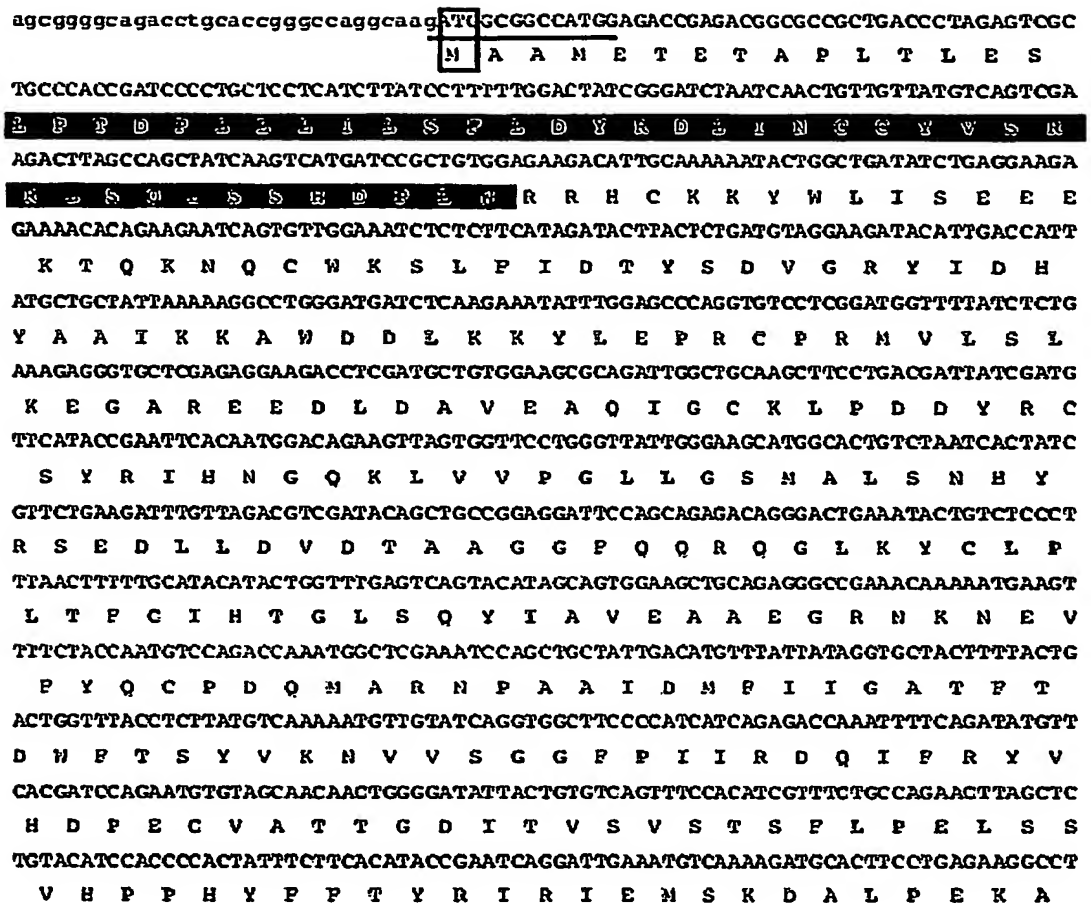

ausgeschnittene Proteinbanden
1
kDa:
anti-PSTAIRE
CDK1/ CDK2/ CDK3
31-34
anti-Cyclin A1
Cyclin A1
56
anti-Cyclin E1
Cyclin E1
46
anti-Cyclin B1
Cyclin B1
60
anti-p19SKP1
p19SKP1
19

Fig. 4.5a: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M A A M E T E T A P L T L E S
TGCCCACCGATCCCCTGCTCCTCATCTTATCCTTTTTGGACTATCGGGATCTAATCAACTGTTGTTATGTCAGTCGA
AGACTTAGCCAGCTATCAAGTCATGATCCGCTGTGGAGAAGACATTGCAAAAAATACTGGCTGATATCTGAGGAAGA
R R H C K K Y W L I S E E E
GAAAACACAGAAGAATCAGTGTTGGAAATCTCTCTTCATAGATACTTACTCTGATGTAGGAAGATACATTGACCATT
K T Q K N Q C W K S L P I D T Y S D V G R Y I D H
ATGCTGCTATTAAAAAGGCCTGGGATGATCTCAAGAAATATTTGGAGCCCAGGTGTCCTCGGATGGTTTTATCTCTG
Y A A I K K A W D D L K K Y L E P R C P R M V L S L
AAAGAGGGTGCTCGAGAGGAAGACCTCGATGCTGTGGAAGCGCAGATTGGCTGCAAGCTTCCTGACGATTATCGATG
K E G A R E E D L D A V E A Q I G C K L P D D Y R C
TTCATACCGAATTCACAATGGACAGAAGTTAGTGGTTCCTGGGTTATTGGGAAGCATGGCACTGTCTAATCACTATC
S Y R I H N G Q K L V V P G L L G S M A L S N H Y
GTTCTGAAGATTTGTTAGACGTCGATACAGCTGCCGGAGGATTCCAGCAGAGACAGGGACTGAAATACTGTCTCCCT
R S E D L L D V D T A A G G P Q Q R Q G L K Y C L P
TTAACTTTTTGCATACATACTGGTTTGAGTCAGTACATAGCAGTGGAAGCTGCAGAGGGCCGAAACAAAAATGAAGT
L T P C I H T G L S Q Y I A V E A A E G R N K N E V
TTTCTACCAATGTCCAGACCAAATGGCTCGAAATCCAGCTGCTATTGACATGTTTATTATAGGTGCTACTTTTACTG
P Y Q C P D Q M A R N P A A I D M P I I G A T P T
ACTGGTTTACCTCTTATGTCAAAAATGTTGTATCAGGTGGCTTCCCCATCATCAGAGACCAAATTTTCAGATATGTT
D W P T S Y V K N V V S G G P P I I R D Q I P R Y V
CACGATCCAGAATGTGTAGCAACAACTGGGGATATTACTGTGTCAGTTCCACATCGTTTCTGCCAGAACTTAGCTC
H D P E C V A T T G D I T V S V S T S P L P E L S S
TGTACATCCACCCCACTATTTCTTCACATACCGAATCAGGATTGAAATGTCAAAAGATGCACTTCCTGAGAAGGCCT
V H P P H Y P P T Y R I R I E M S K D A L P E K A

Fig. 4.5b:

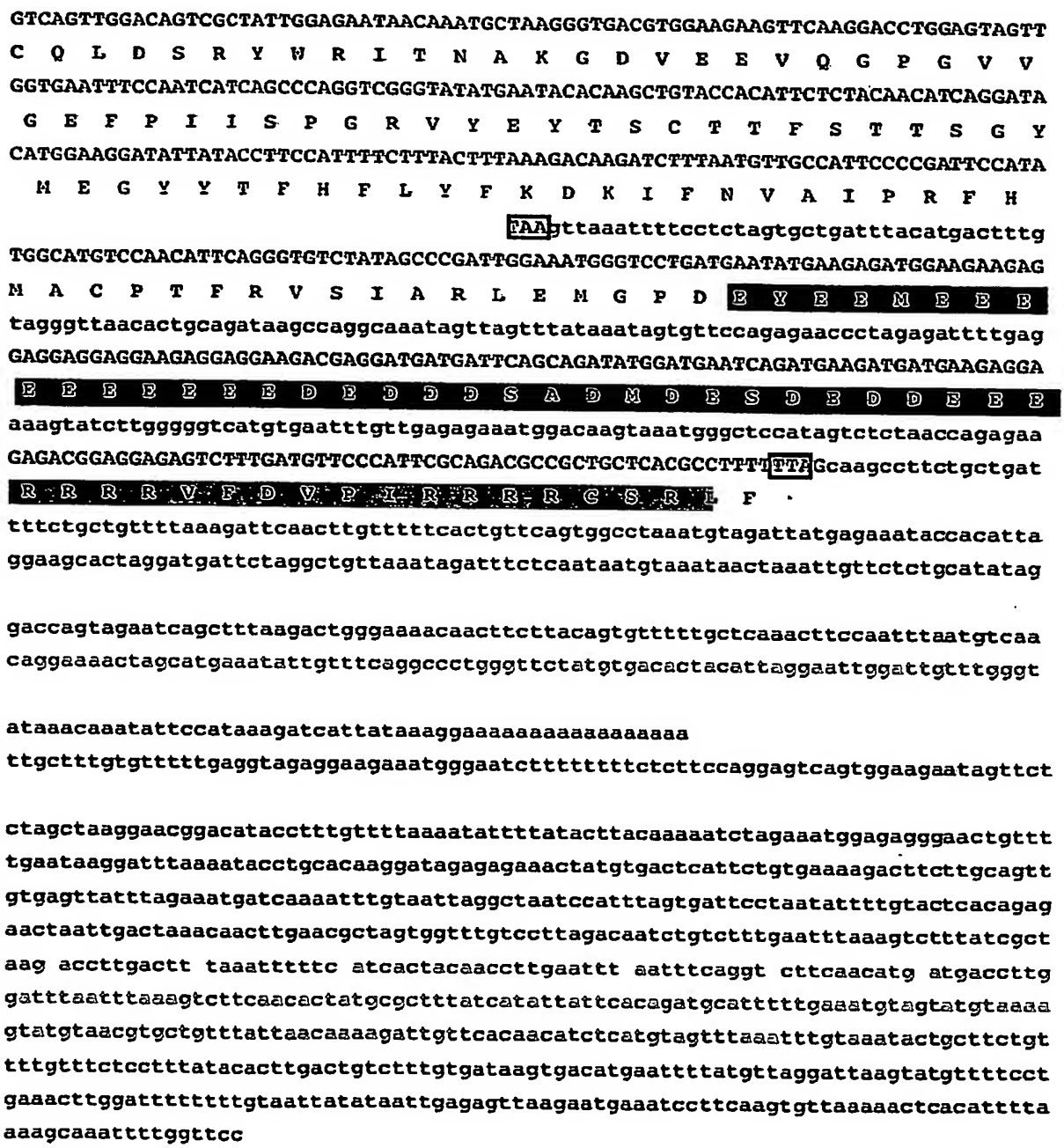

GTCAGTTGGACAGTCGCTATTGGAGAATAACAAATGCTAAGGGTGACGTGGAAGAAGTTCAAGGACCTGGAGTAGTT
C Q L D S R Y W R I T N A K G D V E E V Q G P G V V
GGTGAATTTCCAATCATCAGCCCAGGTCGGGTATATGAATACACAAGCTGTACCACATTCTCTACAACATCAGGATA
G E F P I I S P G R V Y E Y T S C T T F S T T S G Y
CATGGAAGGATATTATACCTTCCATTTTCTTTACTTTAAAGACAAGATCTTTAATGTTGCCATTCCCCGATTCCATA
M E G Y Y T F H F L Y F K D K I F N V A I P R F H
TAAgttaaattttcctctagtgctgatttacatgactttg
TGGCATGTCCAACATTCAGGGTGTCTATAGCCCGATTGGAAATGGGTCCTGATGAATATGAAGAGATGGAAGAAGAG
M A C P T F R V S I A R L E M G P D E Y E E M E E E
tagggttaacactgcagataagccaggcaaatagttagtttataaatagtgttccagagaaccctagagattttgag
GAGGAGGAGGAAGAGGAGGAAGACGAGGATGATGATTCAGCAGATATGGATGAATCAGATGAAGATGATGAAGAGGA
E E E E E E E D E D D D S A D M D E S D E D D E E E
aaagtatcttgggggtcatgtgaatttgttgagagaaatggacaagtaaatgggctccatagtctctaaccagagaa
GAGACGGAGGAGAGTCTTTGATGTTCCCATTCGCAGACGCCGCTGCTCACGCCTTTTTTAGcaagccttctgctgat
R R R R V F D V P I R R R R C S R L F
tttctgctgttttaaagattcaacttgtttttcactgttcagtggcctaaatgtagattatgagaaataccacatta
ggaagcactaggatgattctaggctgttaaatagatttctcaataatgtaaataactaaattgttctctgcatatag
gaccagtagaatcagctttaagactgggaaaacaacttcttacagtgttttgctcaaacttccaatttaatgtcaa
caggaaaactagcatgaaatattgtttcaggccctgggttctatgtgacactacattaggaattggattgtttgggt
ataaacaaatattccataaagatcattataaaggaaaaaaaaaaaaaaaa
ttgctttgtgtttttgaggtagaggaagaaatgggaatctttttttttctcttccaggagtcagtggaagaatagttct
ctagctaaggaacggacatacctttgttttaaaatattttatacttacaaaaatctagaaatggagagggaactgttt
tgaataaggatttaaaatacctgcacaaggatagagagaaactatgtgactcattctgtgaaaagacttcttgcagtt
gtgagttatttagaaatgatcaaaatttgtaattaggctaatccatttagtgattcctaatattttgtactcacagag
aactaattgactaaacaacttgaacgctagtggtttgtccttagacaatctgtctttgaatttaaagtctttatcgct
aag accttgactt taaatttttc atcactacaaccttgaattt aatttcaggt cttcaacatg atgaccttg
gatttaatttaaagtcttcaacactatgcgctttatcatattattcacagatgcatttttgaaatgtagtatgtaaaa
gtatgtaacgtgctgtttattaacaaaagattgttcacaacatctcatgtagtttaaatttgtaaatactgcttctgt
tttgtttctcctttatacacttgactgtctttgtgataagtgacatgaattttatgttaggattaagtatgttttcct
gaaacttggatttttttgtaattatataattgagagttaagaatgaaatccttcaagtgttaaaaactcacatttta
aaagcaaattttggttcc

Fig. 4.6:

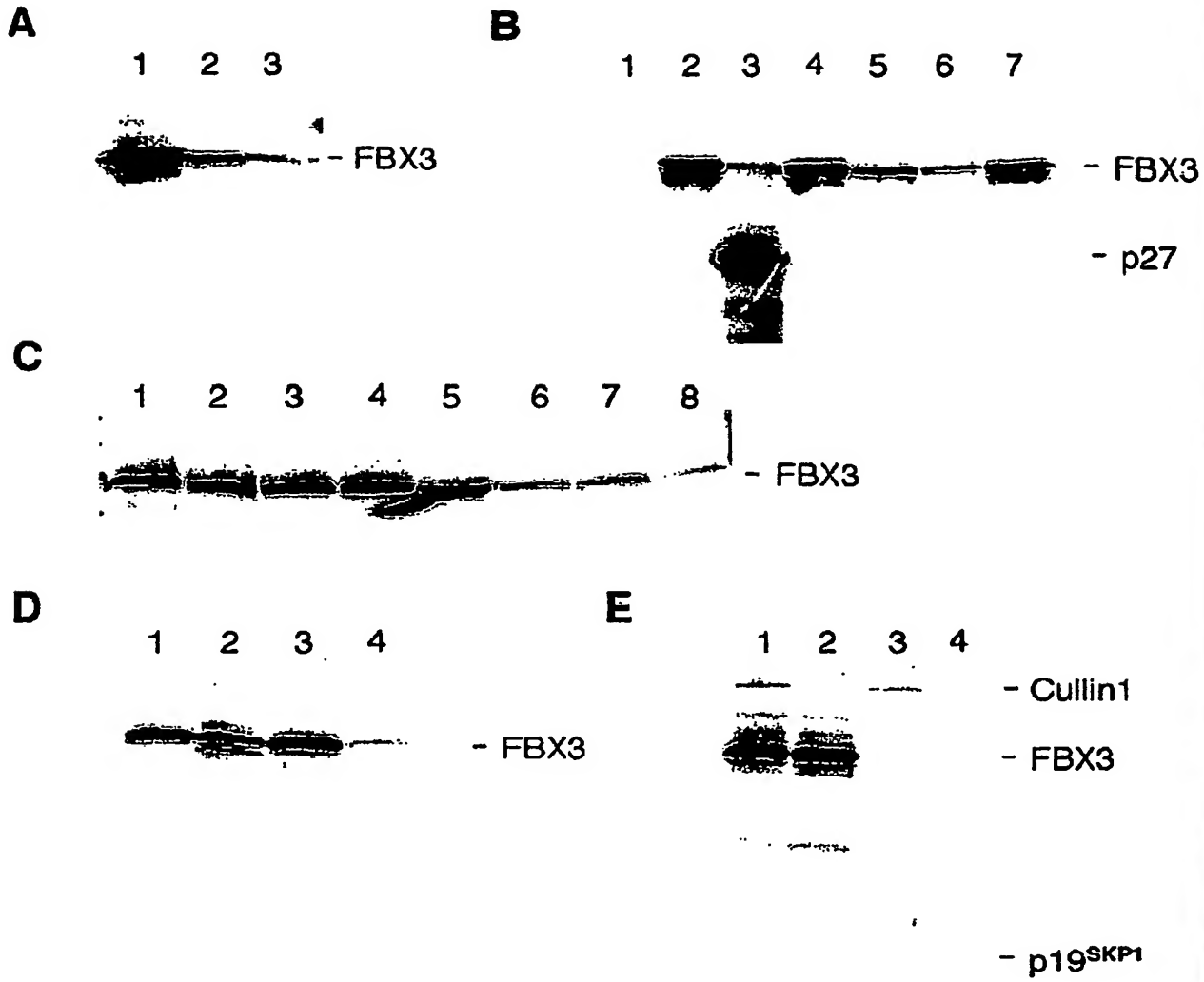

A
1 2 3
FBX3
B
1 2 3 4 5 6 7
FBX3
p27
C
1 2 3 4 5 6 7 8
FBX3
D
1 2 3 4
FBX3
E
1 2 3 4
Cullin1
FBX3
p19SKP1

Fig. 4.7:

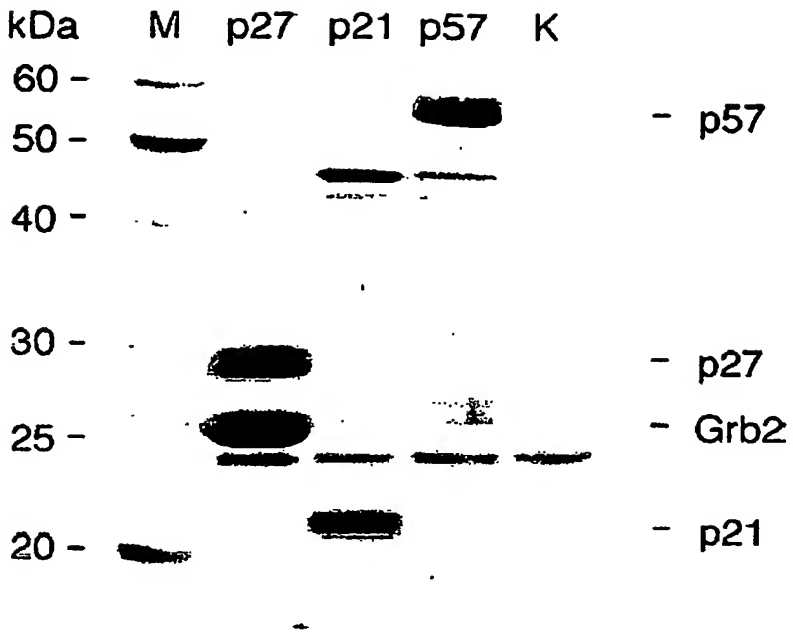


Fig. 4.8.:

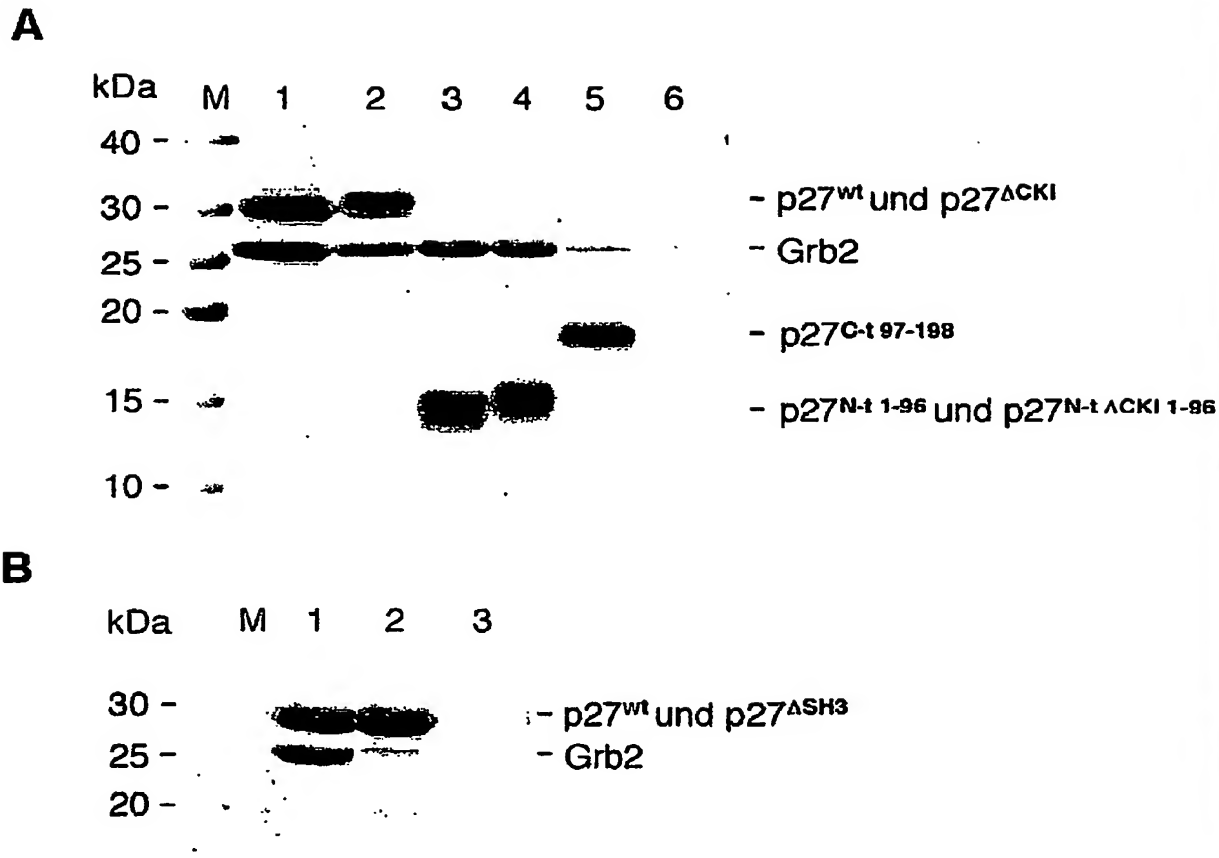

A
kDa
M
1
2
3
4
5
6
40 -
30 -
25 -
20 -
15 -
10 -
- p27wt und p27ΔCKI
- Grb2
- p27C-t 97-198
- p27N-t 1-96 und p27N-t ΔCKI 1-96
B
kDa
M
1
2
3
30 -
25 -
20 -
- p27wt und p27ΔSH3
- Grb2

Fig. 4.9:

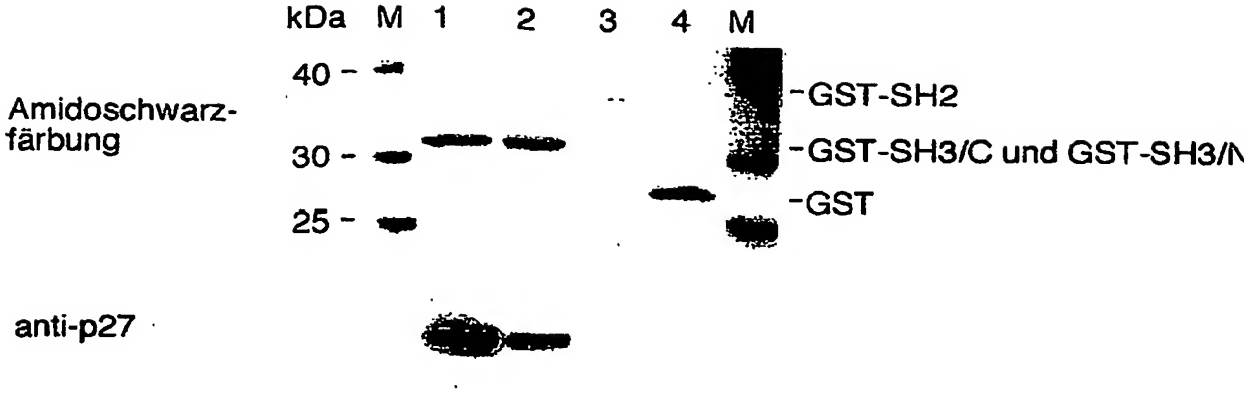

kDa
M
1
2
3
4
M
40 -
30 -
25 -
Amidoschwarz-
färbung
-GST-SH2
-GST-SH3/C und GST-SH3/N
-GST
anti-p27

Fig. 4.10:

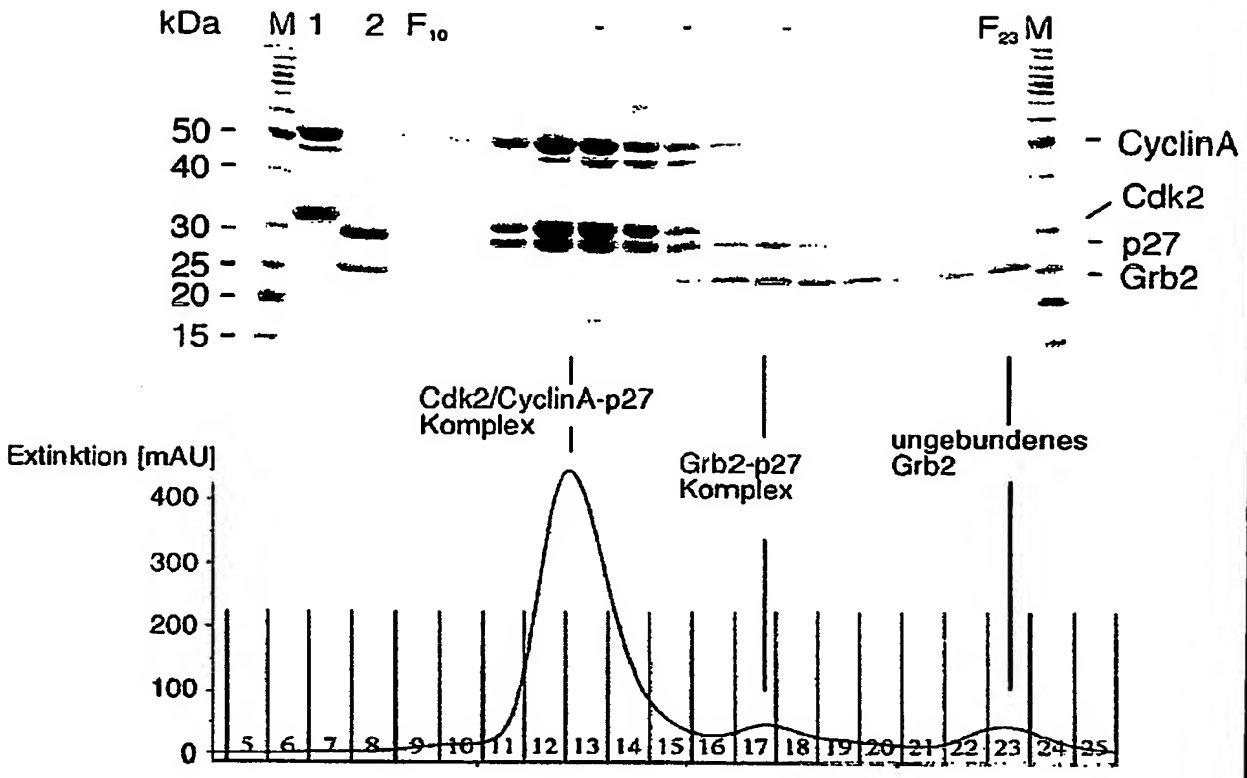
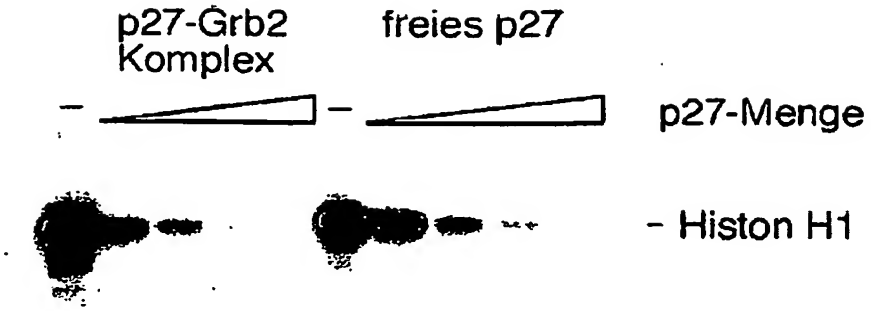

kDa
M 1 2 F10 - - - F23 M
50
40
30
25
20
15
CyclinA
Cdk2
p27
Grb2
Cdk2/CyclinA-p27 Komplex
Grb2-p27 Komplex
ungebundenes Grb2
Extinktion [mAU]
400
300
200
100
0
5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

Fig. 4.11:



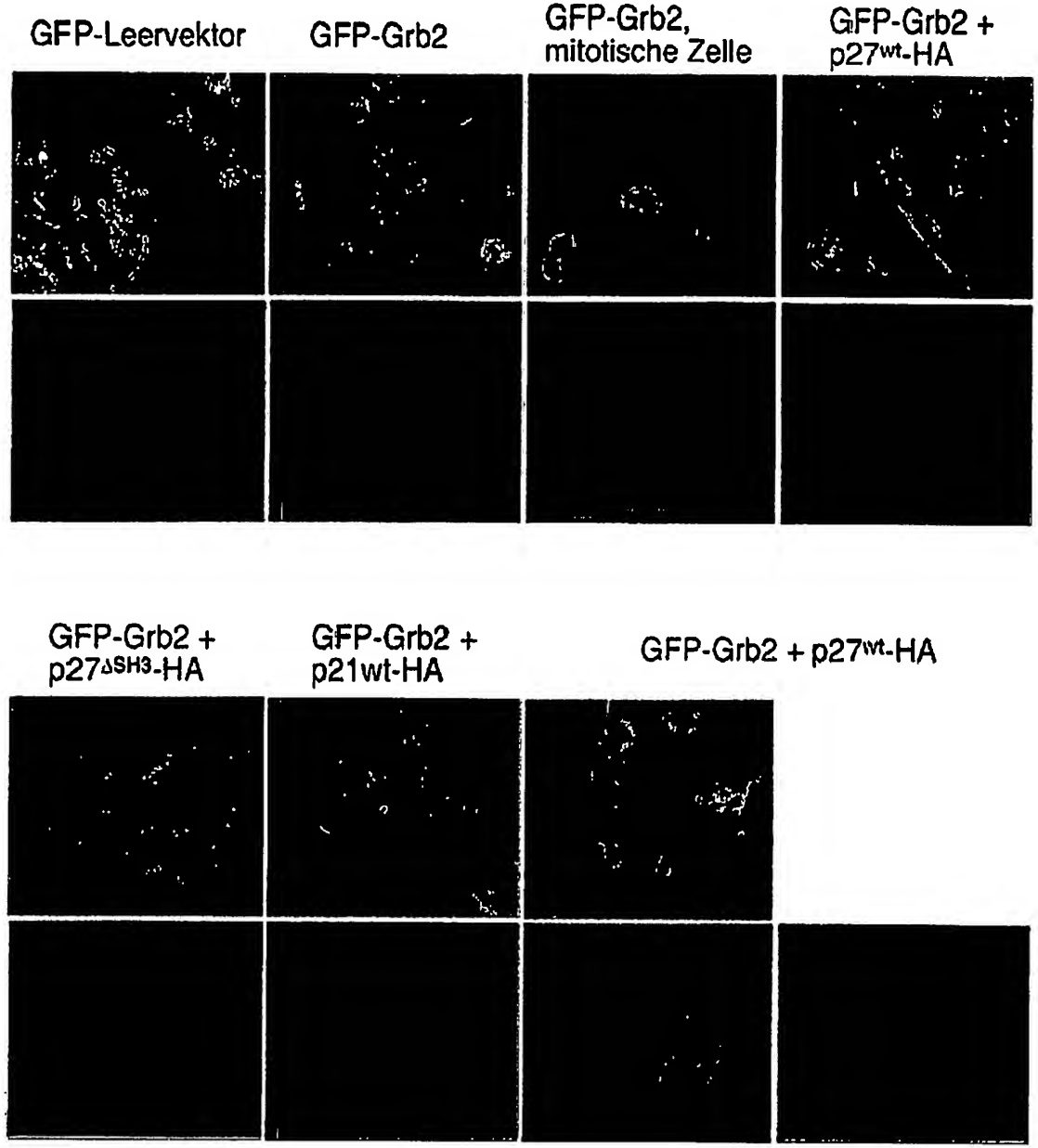


Fig. 4.12:

Abbildung 4
Lokalisation
und Lokalisa
Grb2 kotransf
Zur Untersuch
Grb2 wurde G
mit GFP kl
Transfektion v
wurde die L
Fluoreszenzli
mikroskopisch
diente der C
untersuchen
Lokalisation
verursachen ka
p27-HA kotran
nach der Tra
untersucht. Al
ein HA-Fusion
HA-Fusionsl
domänenbindu
$p27^{\Delta SH3}$. Zu
Transfektions
mittels spezifi
Rodamin-Fluor

Fig. 4.13:

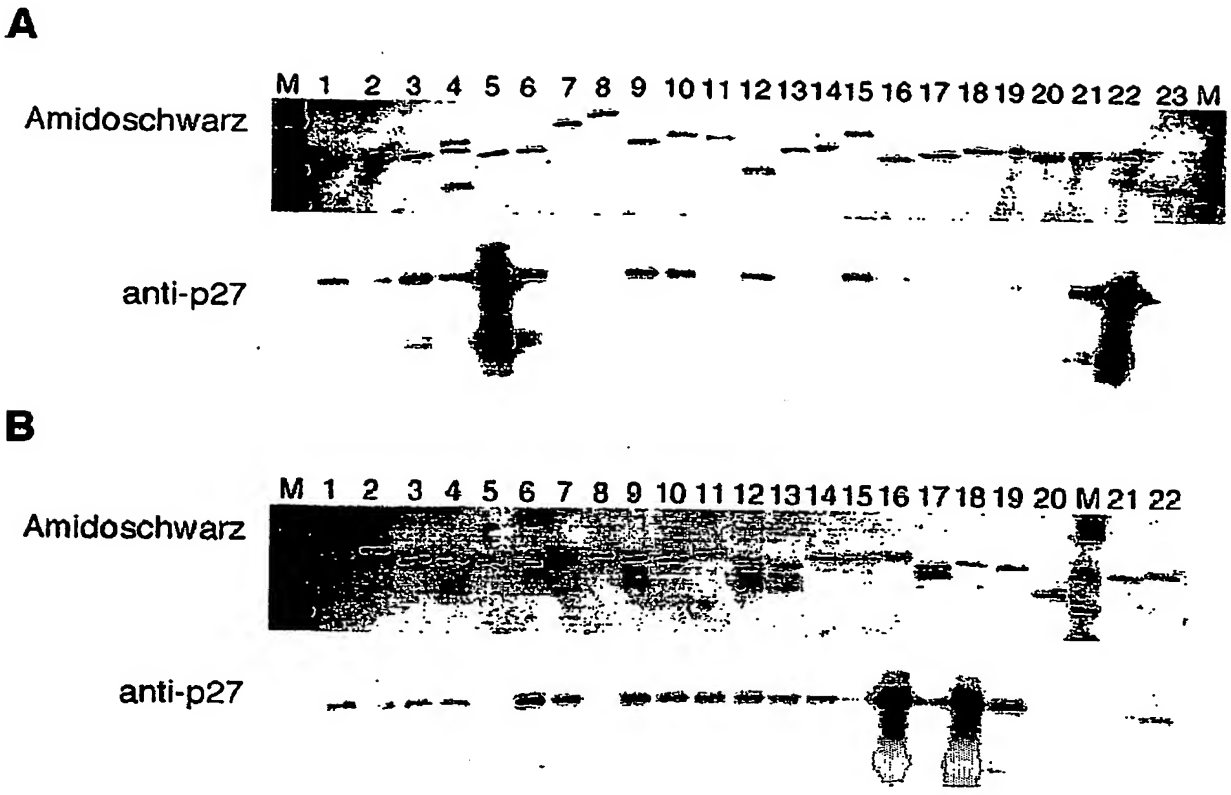

A
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 M
Amidoschwarz
anti-p27
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 M 21 22
Amidoschwarz
anti-p27

Fig. 4.14:

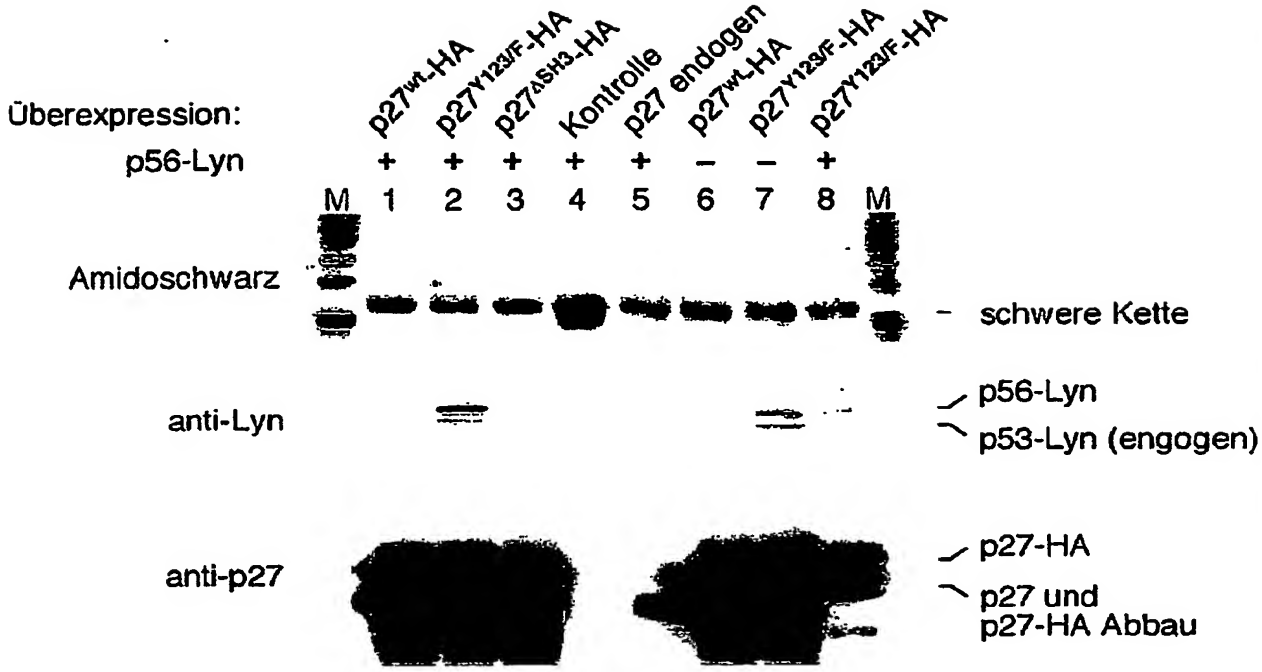

Überexpression:
p56-Lyn
p27wt-HA
p27Y123/F-HA
p27ΔSH3-HA
Kontrolle
p27 endogen
p27wt-HA
p27Y123/F-HA
p27Y123/F-HA
+ + + + + – – +
M 1 2 3 4 5 6 7 8 M
Amidoschwarz
schwere Kette
anti-Lyn
p56-Lyn
p53-Lyn (engogen)
anti-p27
p27-HA
p27 und
p27-HA Abbau

Fig. 4.15

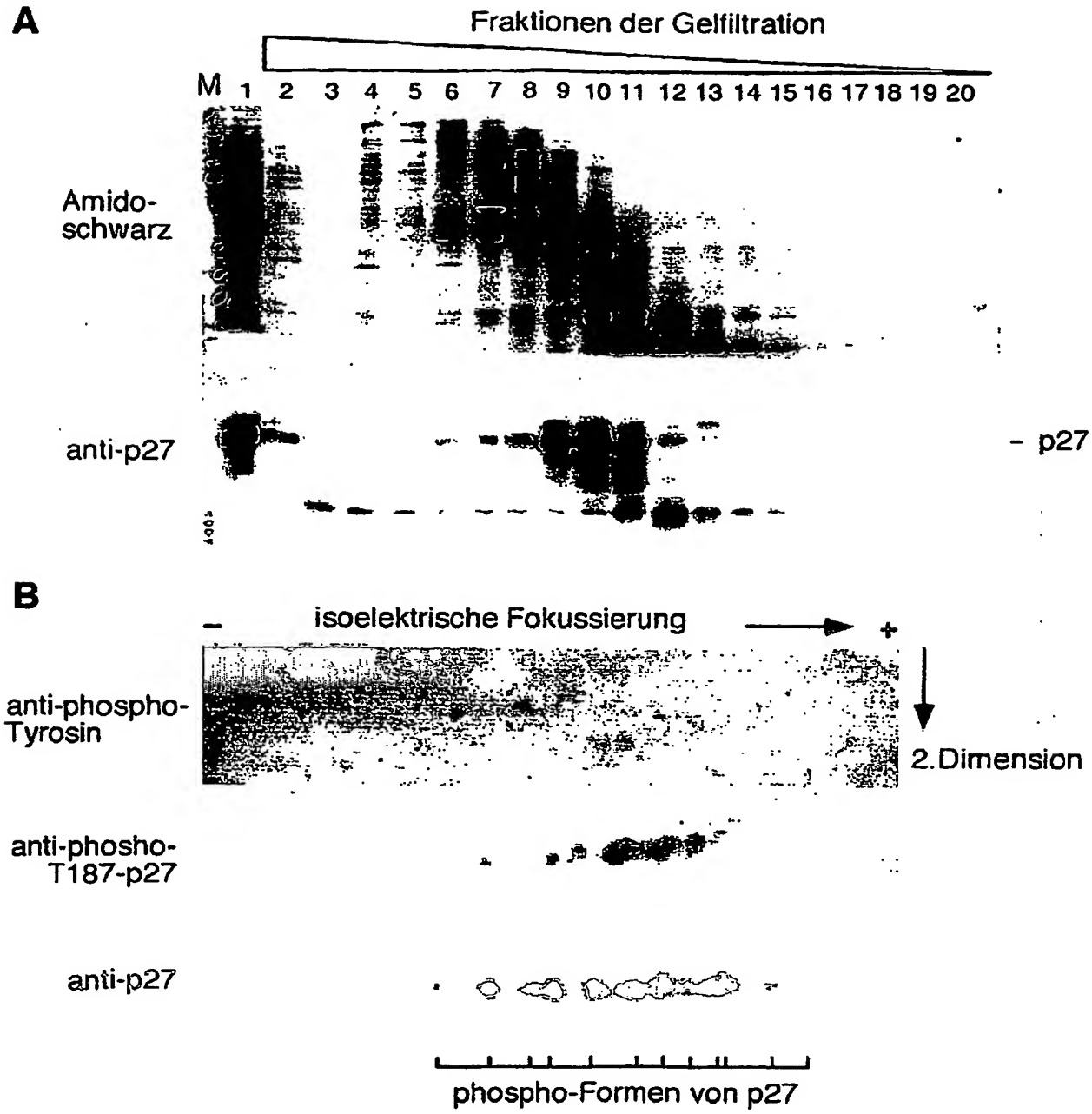

A
Fraktionen der Gelfiltration
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Amido-schwarz
anti-p27
- p27
B
isoelektrische Fokussierung
anti-phospho-Tyrosin
2.Dimension
anti-phosho-T187-p27
anti-p27
phospho-Formen von p27

Fig. 4.16:

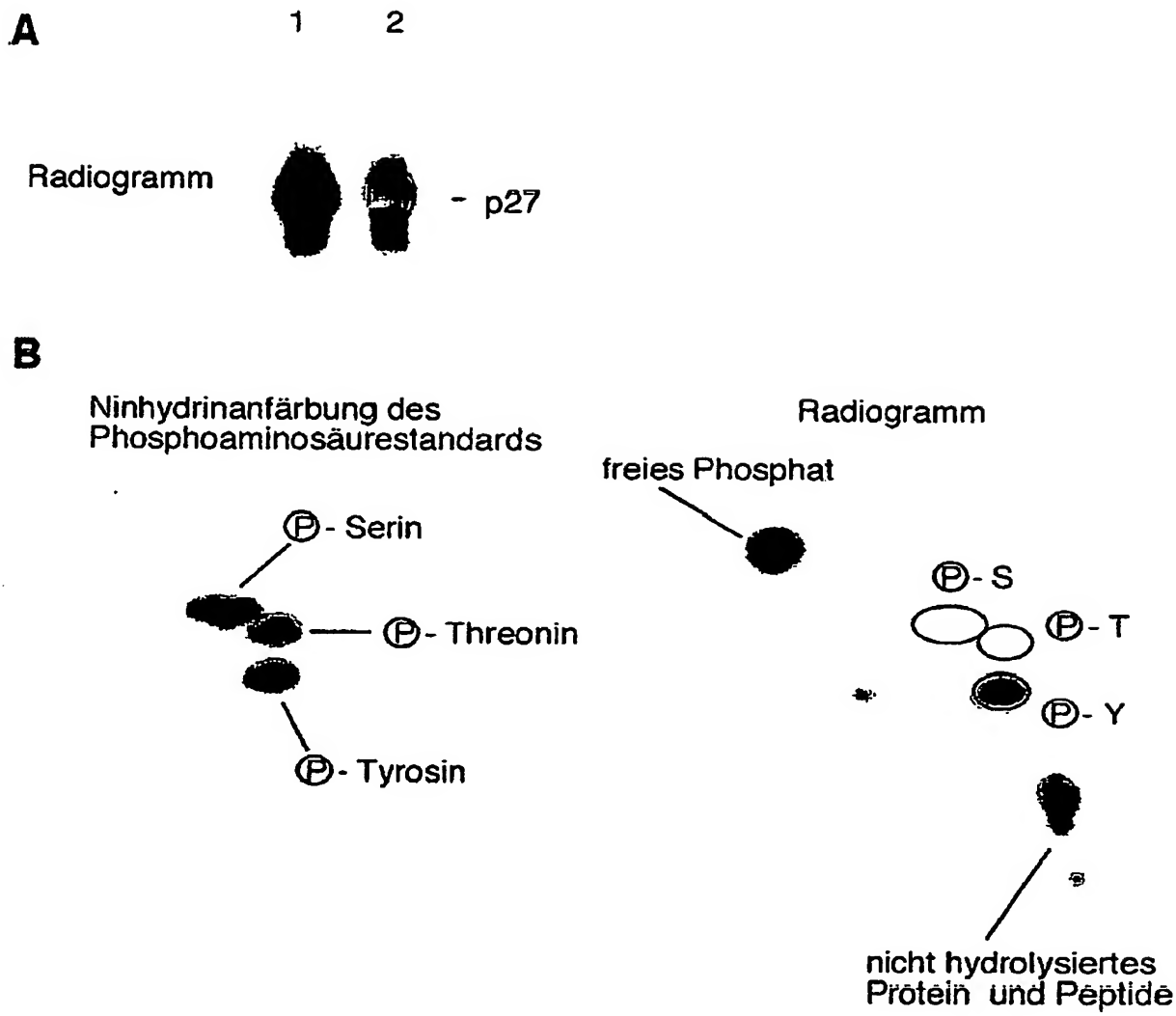

A
1
2
Radiogramm
- p27
B
Ninhydrinanfärbung des
Phosphoaminosäurestandards
Radiogramm
freies Phosphat
Ⓟ- Serin
Ⓟ- Threonin
Ⓟ- Tyrosin
Ⓟ- S
Ⓟ- T
Ⓟ- Y
nicht hydrolysiertes
Protein und Peptide

Fig. 4.17:

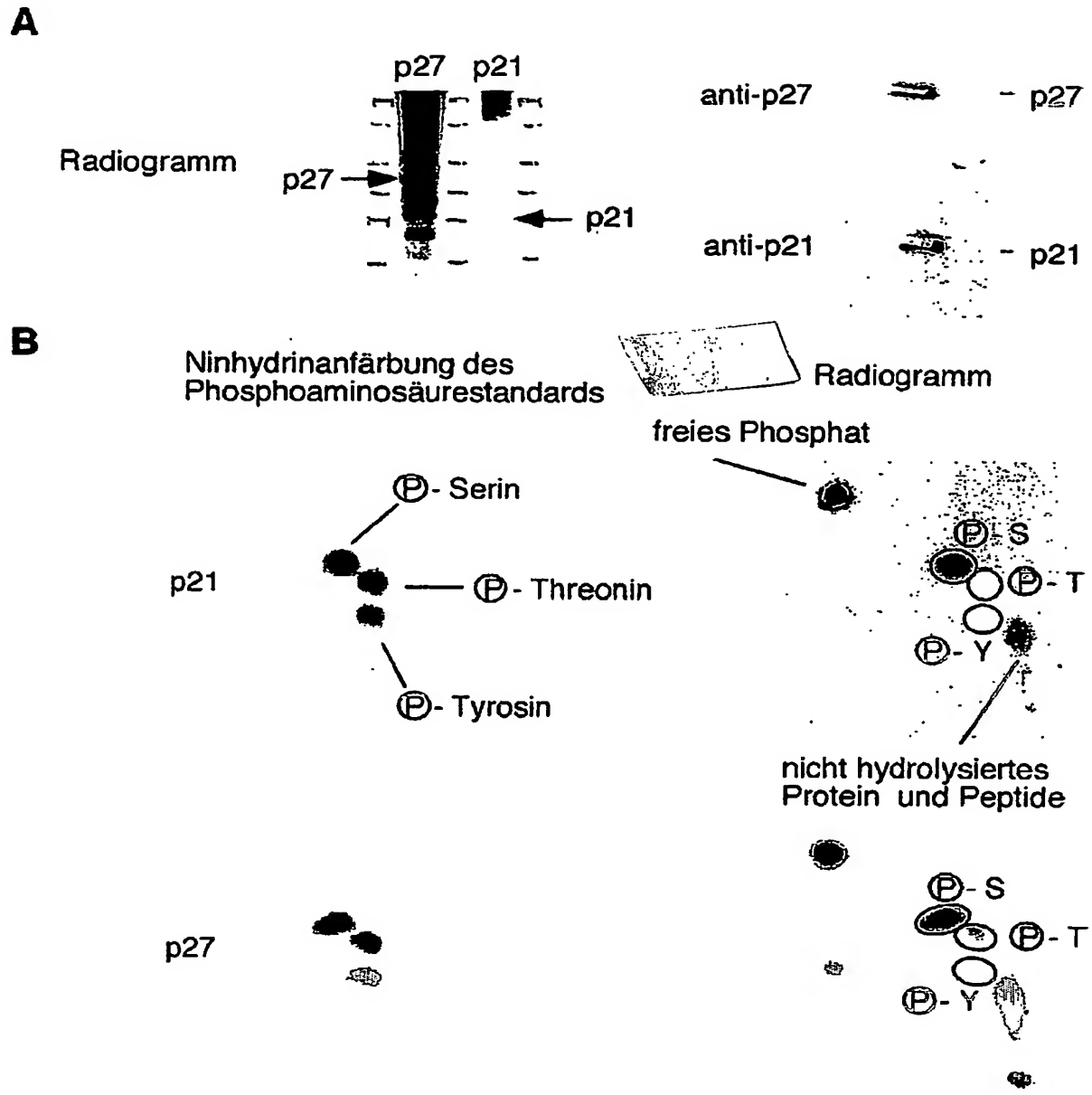

A
p27
p21
Radiogramm
p27
p21
anti-p27
p27
anti-p21
p21
B
Ninhydrinanfärbung des
Phosphoaminosäurestandards
Radiogramm
freies Phosphat
Ⓟ- Serin
p21
Ⓟ- Threonin
Ⓟ- Tyrosin
Ⓟ- S
Ⓟ- T
Ⓟ- Y
nicht hydrolysiertes
Protein und Peptide
p27
Ⓟ- S
Ⓟ- T
Ⓟ- Y

Fig. 4.18:

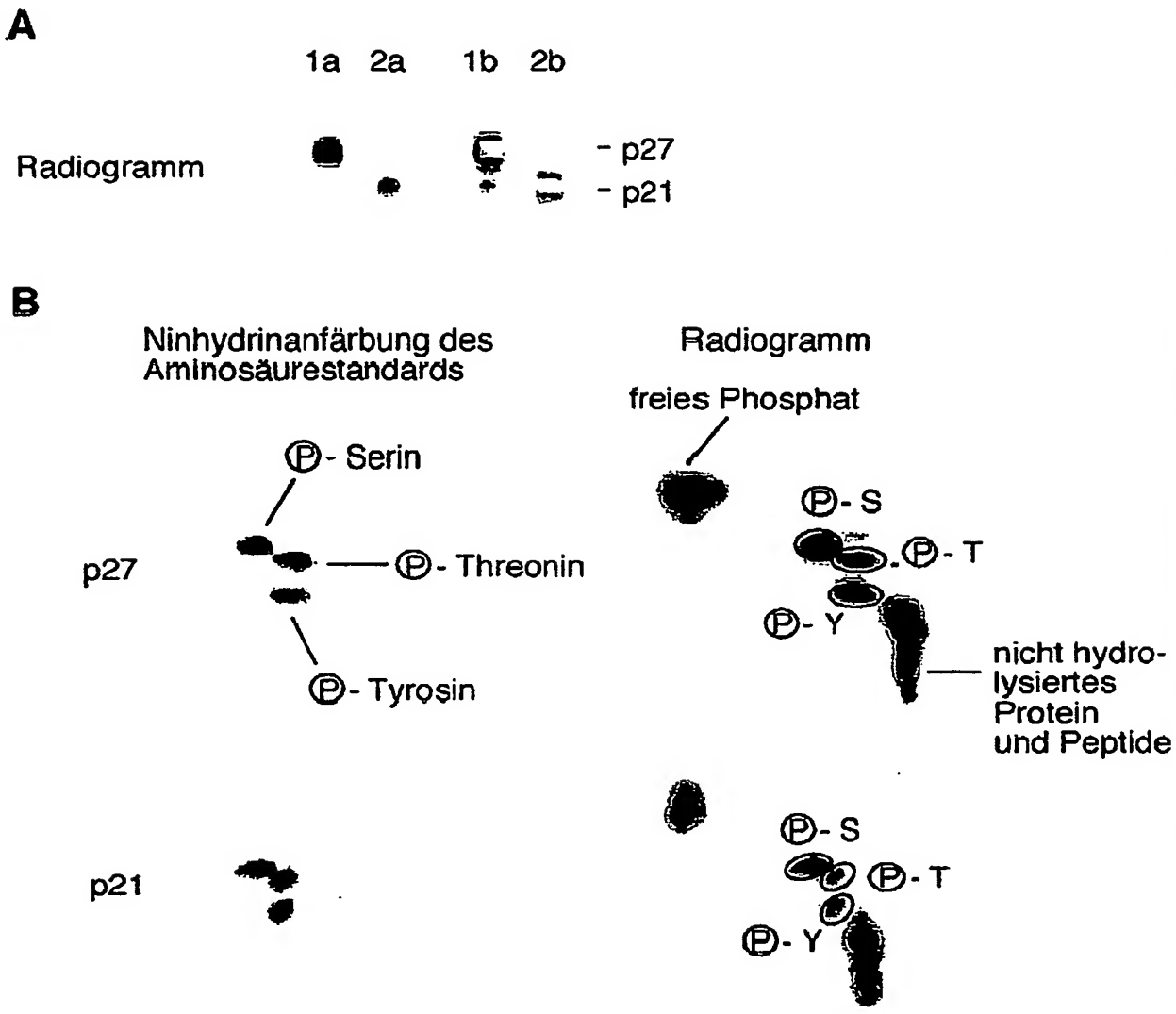

A
1a
2a
1b
2b
Radiogramm
- p27
- p21
B
Ninhydrinanfärbung des Aminosäurestandards
Radiogramm
freies Phosphat
Ⓟ- Serin
p27
Ⓟ- Threonin
Ⓟ- Tyrosin
Ⓟ- S
Ⓟ- T
Ⓟ- Y
nicht hydro-lysiertes Protein und Peptide
p21
Ⓟ- S
Ⓟ- T
Ⓟ- Y

Fig. 4.19:

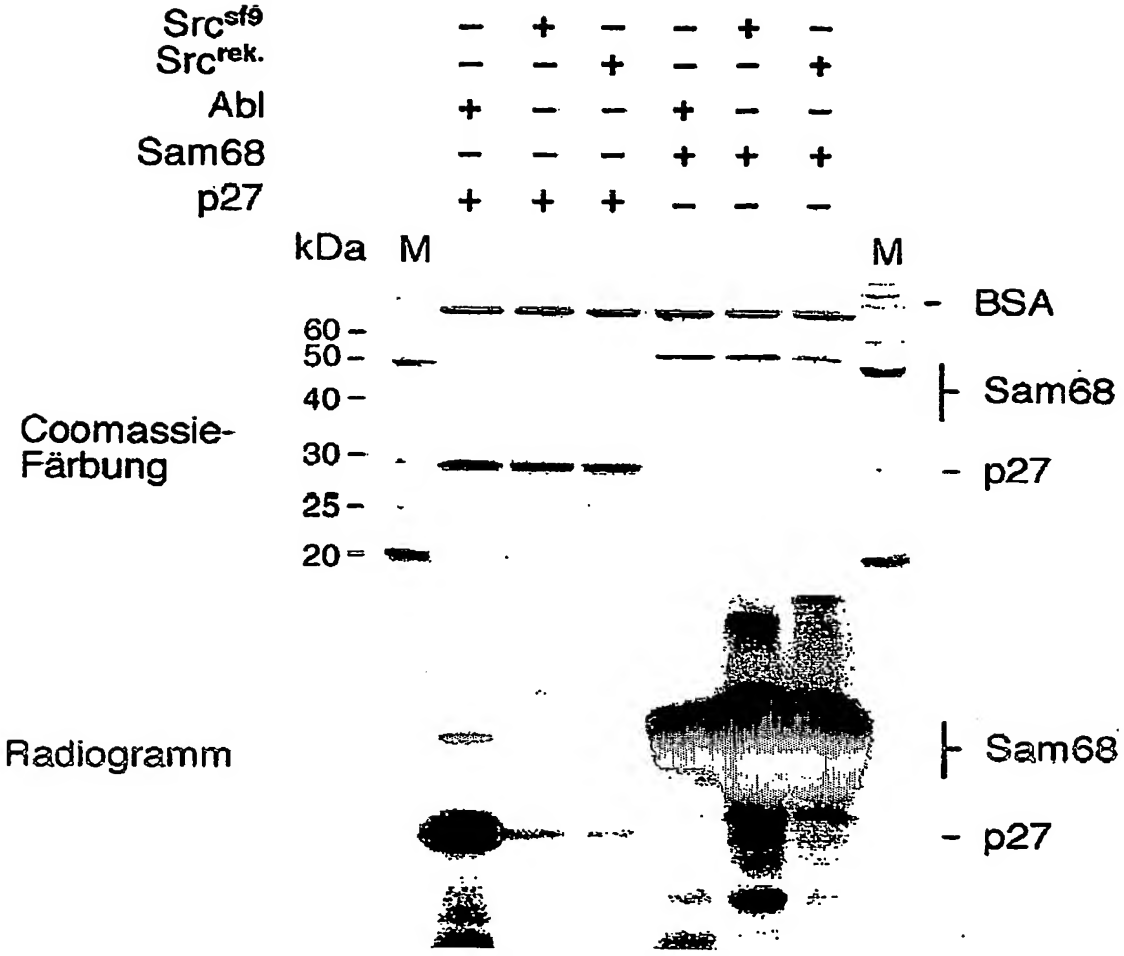

Src^{sf9}
Src$^{rek.}$
Abl
Sam68
p27
kDa
M
M
60
50
40
30
25
20
BSA
Sam68
p27
Coomassie-Färbung
Radiogramm
Sam68
p27

Fig. 4.20:

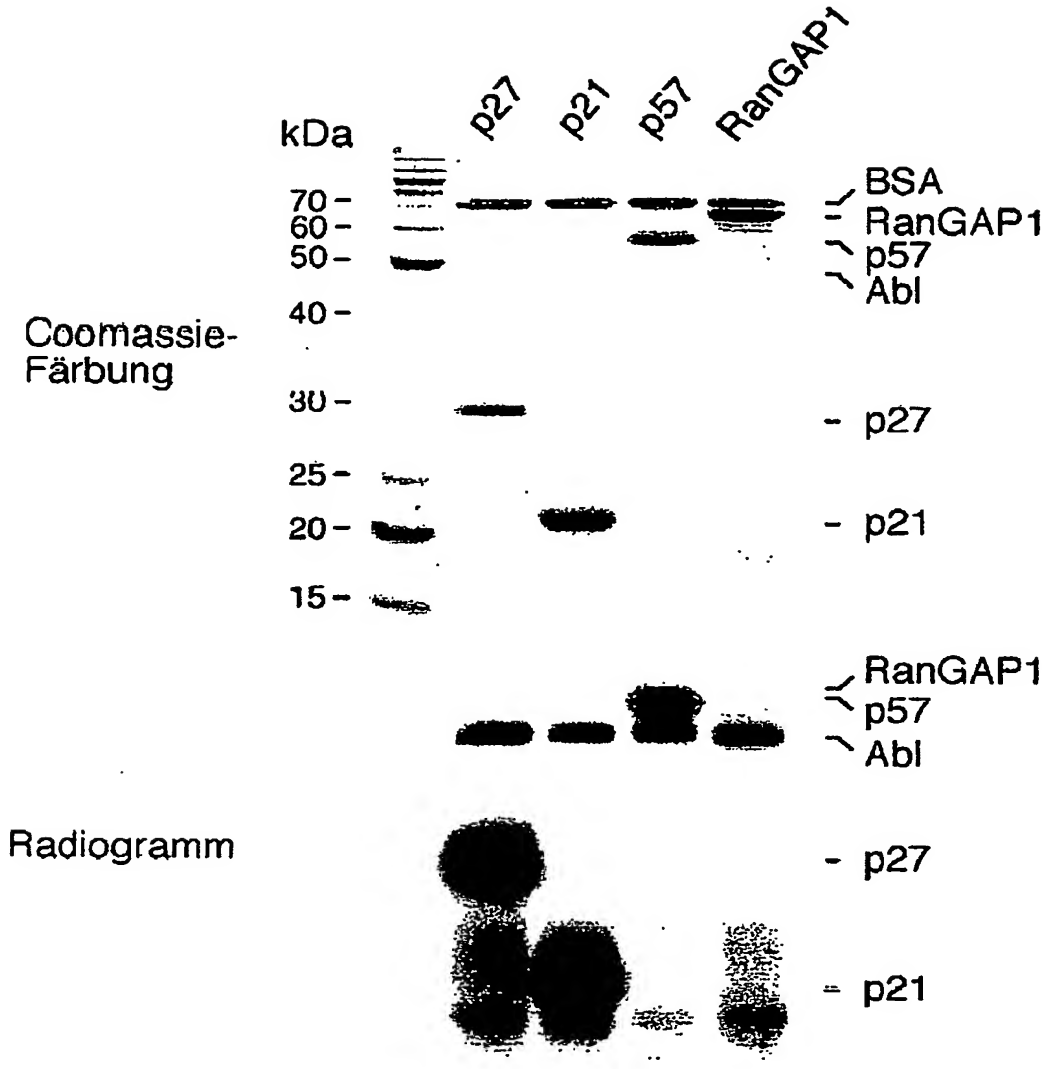

kDa
p27
p21
p57
RanGAP1
70
60
50
40
30
25
20
15
BSA
RanGAP1
p57
Abl
Coomassie-Färbung
- p27
- p21
RanGAP1
p57
Abl
Radiogramm
- p27
- p21

Fig. 4.21:

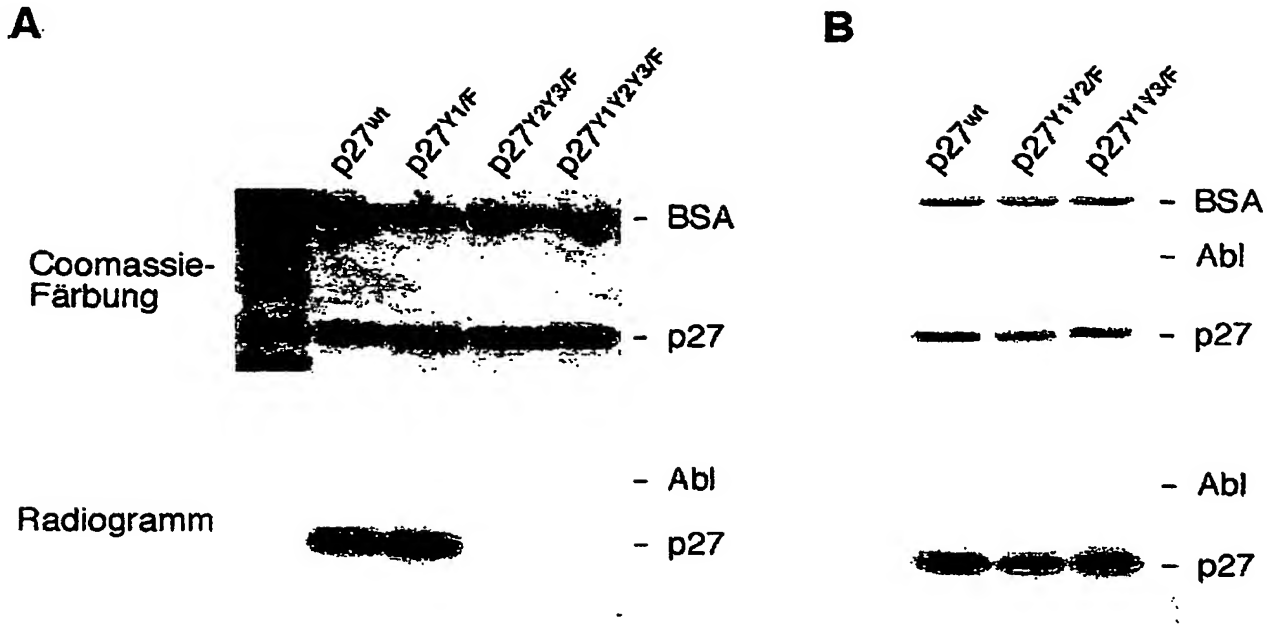

A
p27wt
p27Y1/F
p27Y2Y3/F
p27Y1Y2Y3/F
Coomassie-Färbung
- BSA
- p27
Radiogramm
- Abl
- p27
B
p27wt
p27Y1Y2/F
p27Y1Y3/F
- BSA
- Abl
- p27
- Abl
- p27

Fig. 4.22:

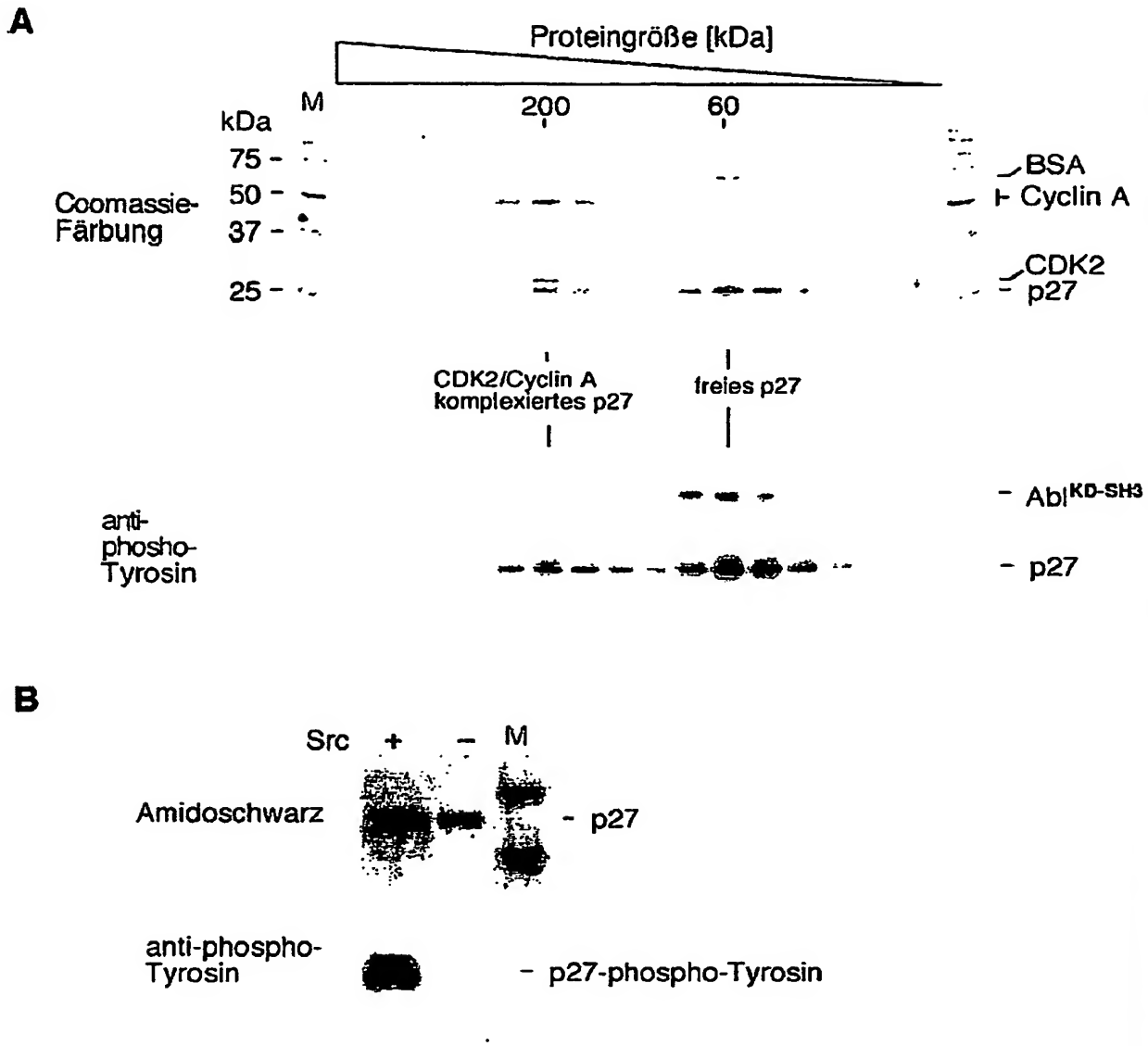

A
Proteingröße [kDa]
M
kDa
200
60
75 -
50 -
37 -
25 -
Coomassie-Färbung
BSA
Cyclin A
CDK2
p27
CDK2/Cyclin A komplexiertes p27
freies p27
Abl KD-SH3
anti-phospho-Tyrosin
p27
B
Src + - M
Amidoschwarz
p27
anti-phospho-Tyrosin
p27-phospho-Tyrosin

Fig. 4.23 A and B:

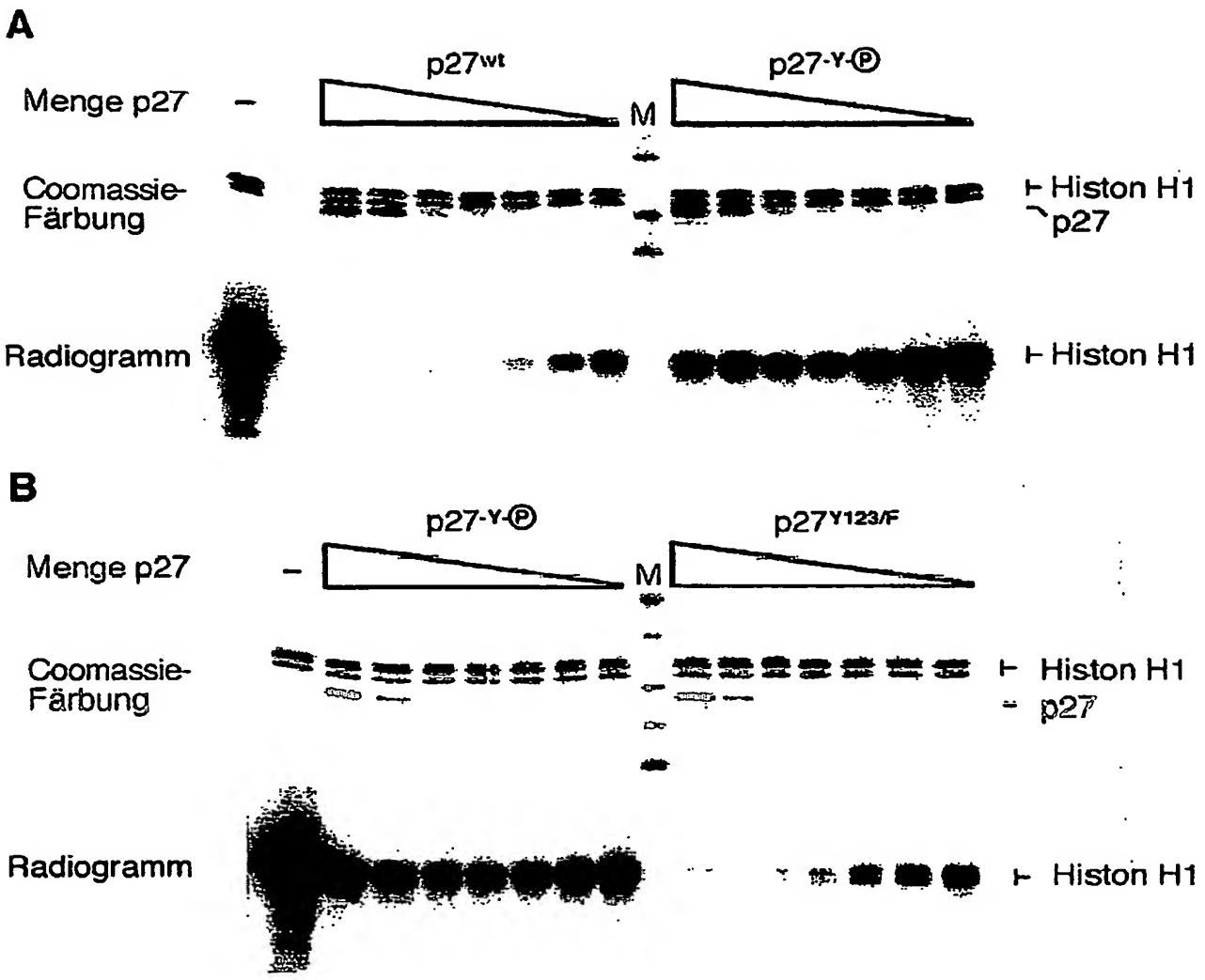


Fig. 4.23 C and D:

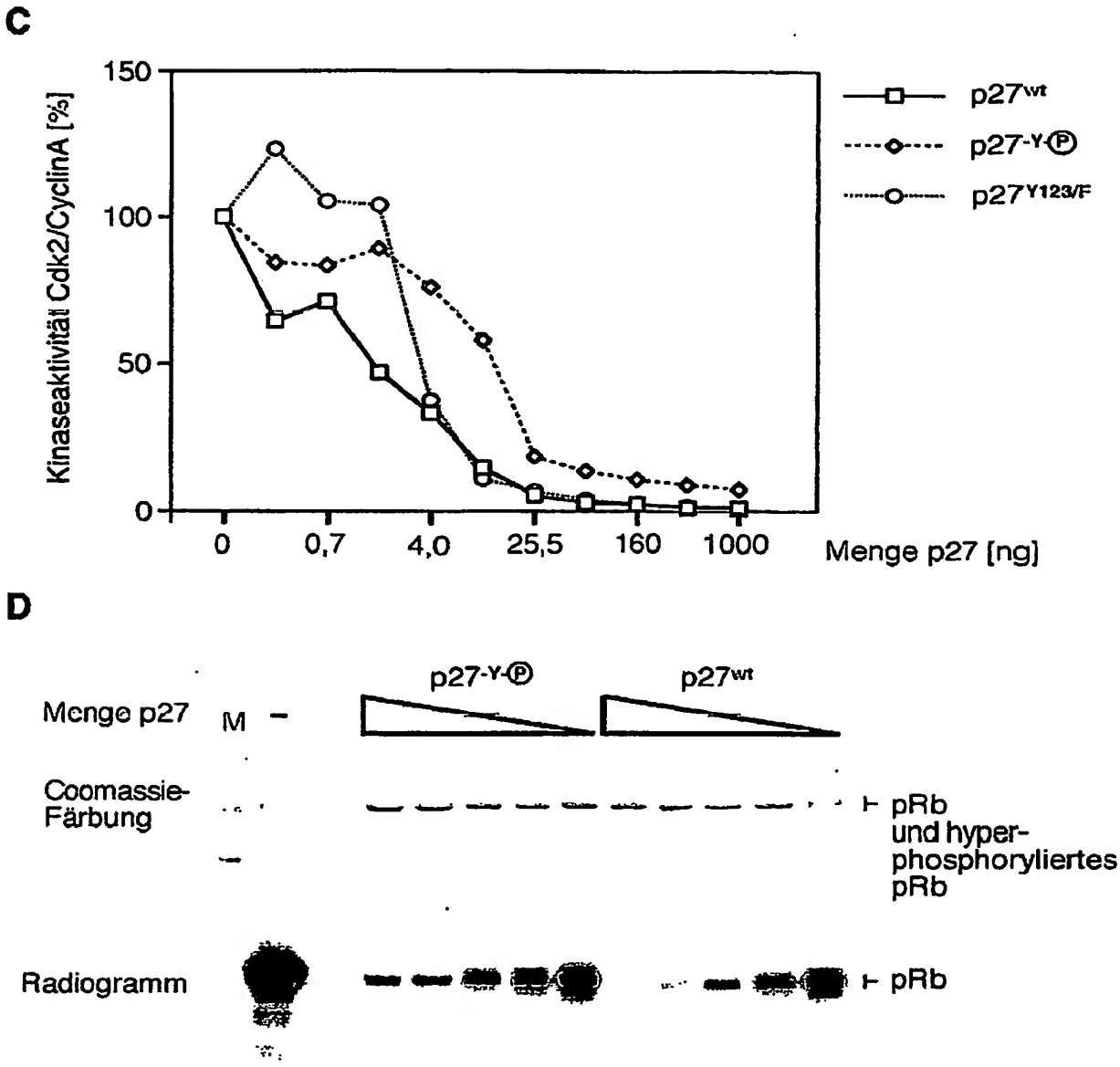


Fig. 4.24:


M
p27wt
p27-Y®
p27Y123F
Histon H1
Coomassie-Färbung
Histon H1
p27
Radiogramm
Histon H1
p27

Fig. 4.25:

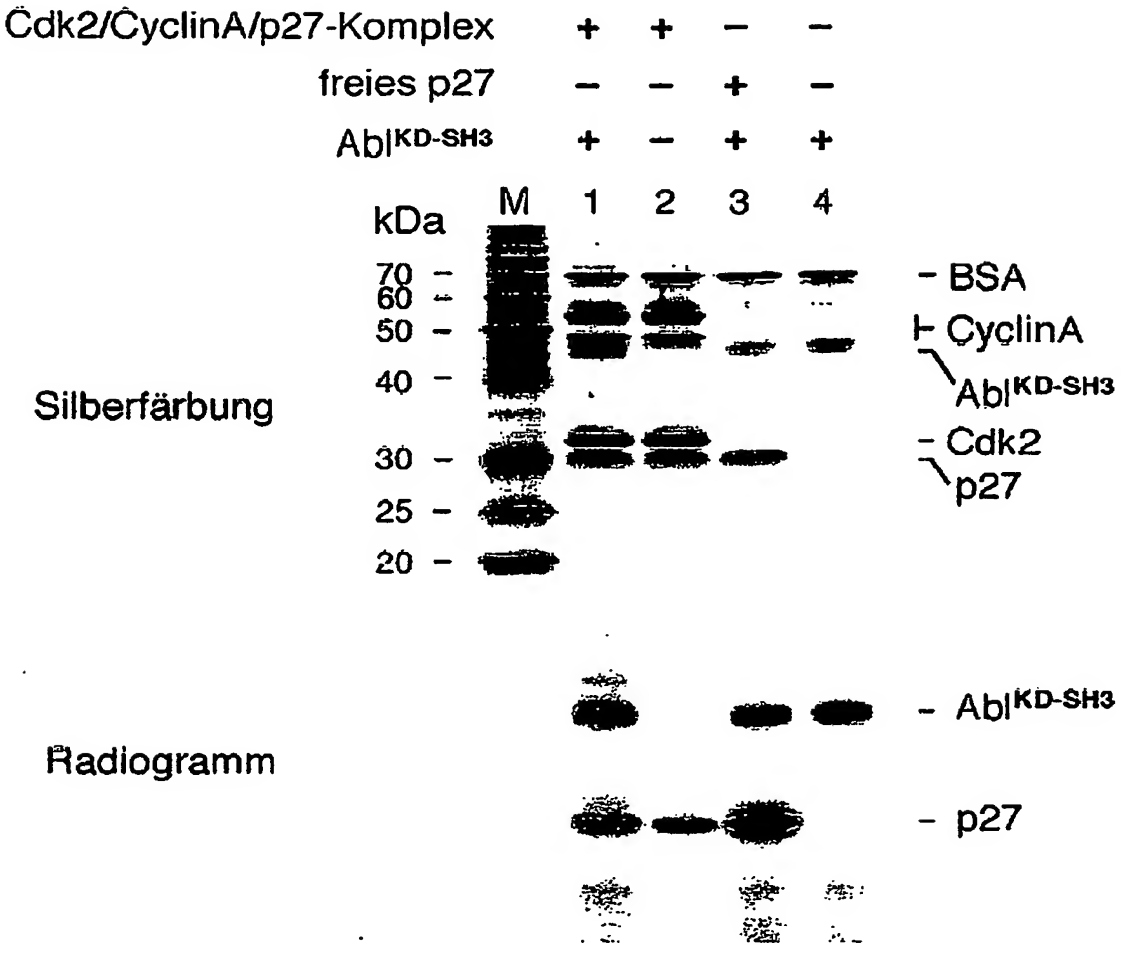

Cdk2/CyclinA/p27-Komplex + + − −
freies p27 − − + −
Abl KD-SH3 + − + +
M 1 2 3 4
kDa
70
60
50
40
30
25
20
Silberfärbung
BSA
CyclinA
Abl KD-SH3
Cdk2
p27
Radiogramm
Abl KD-SH3
p27

Fig. 4.26:

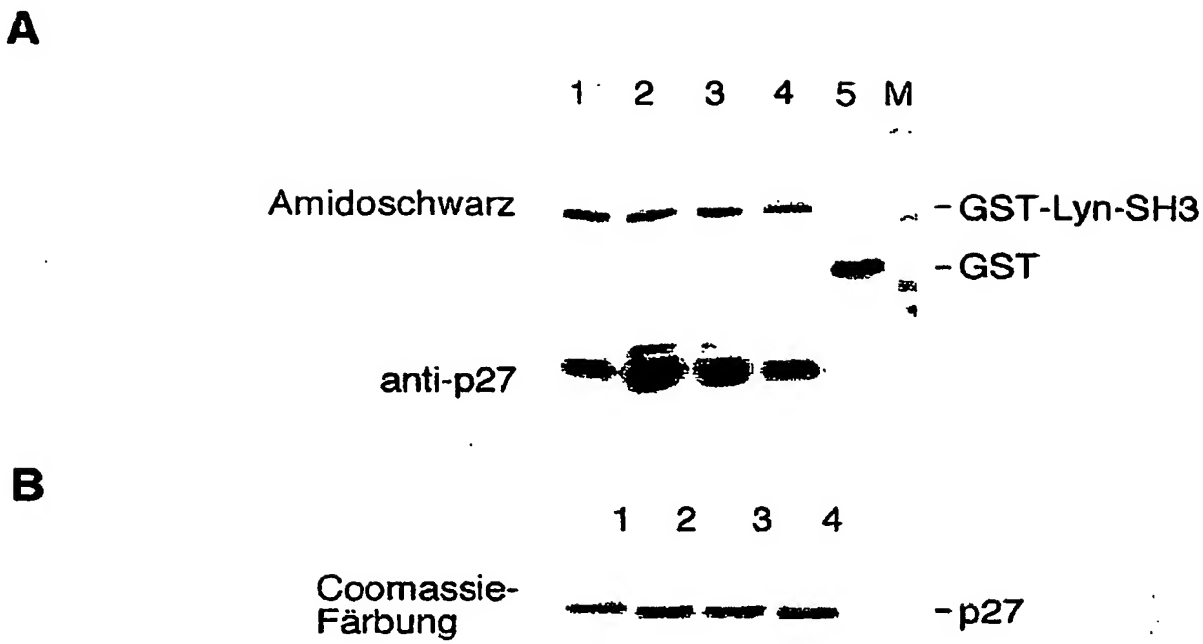

A
1 2 3 4 5 M
Amidoschwarz
- GST-Lyn-SH3
- GST
anti-p27
B
1 2 3 4
Coomassie-Färbung
- p27

Fig. 4.27:

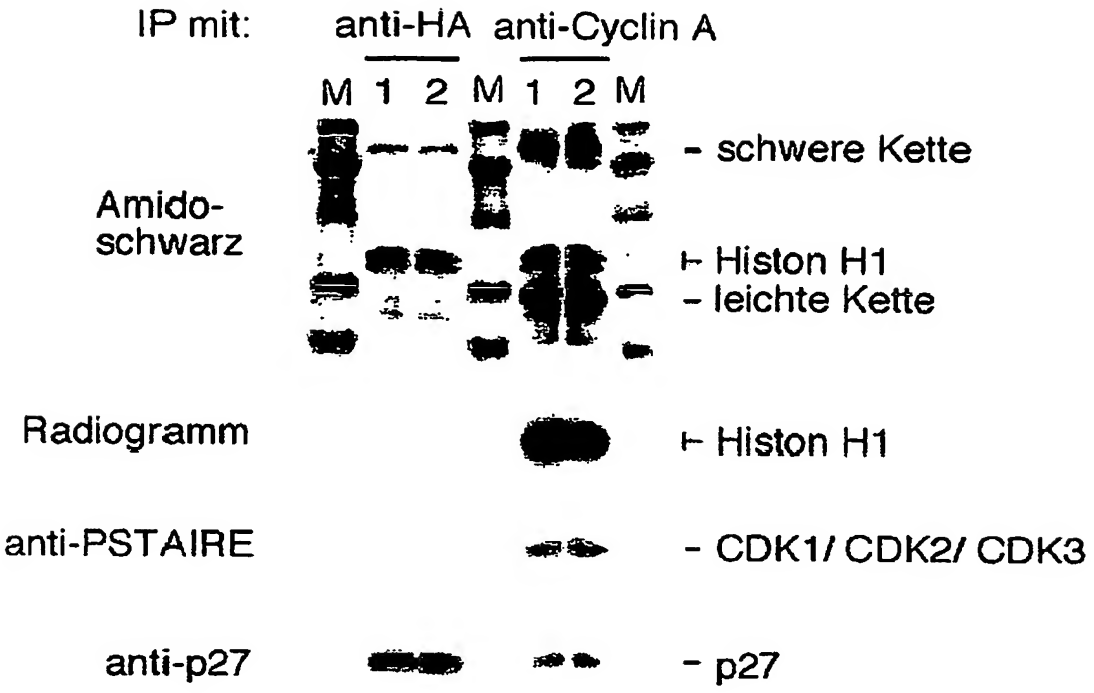

IP mit:
anti-HA
anti-Cyclin A
M 1 2 M 1 2 M
Amido-schwarz
- schwere Kette
⊢ Histon H1
- leichte Kette
Radiogramm
⊢ Histon H1
anti-PSTAIRE
- CDK1/ CDK2/ CDK3
anti-p27
- p27

Fig. 4.28:

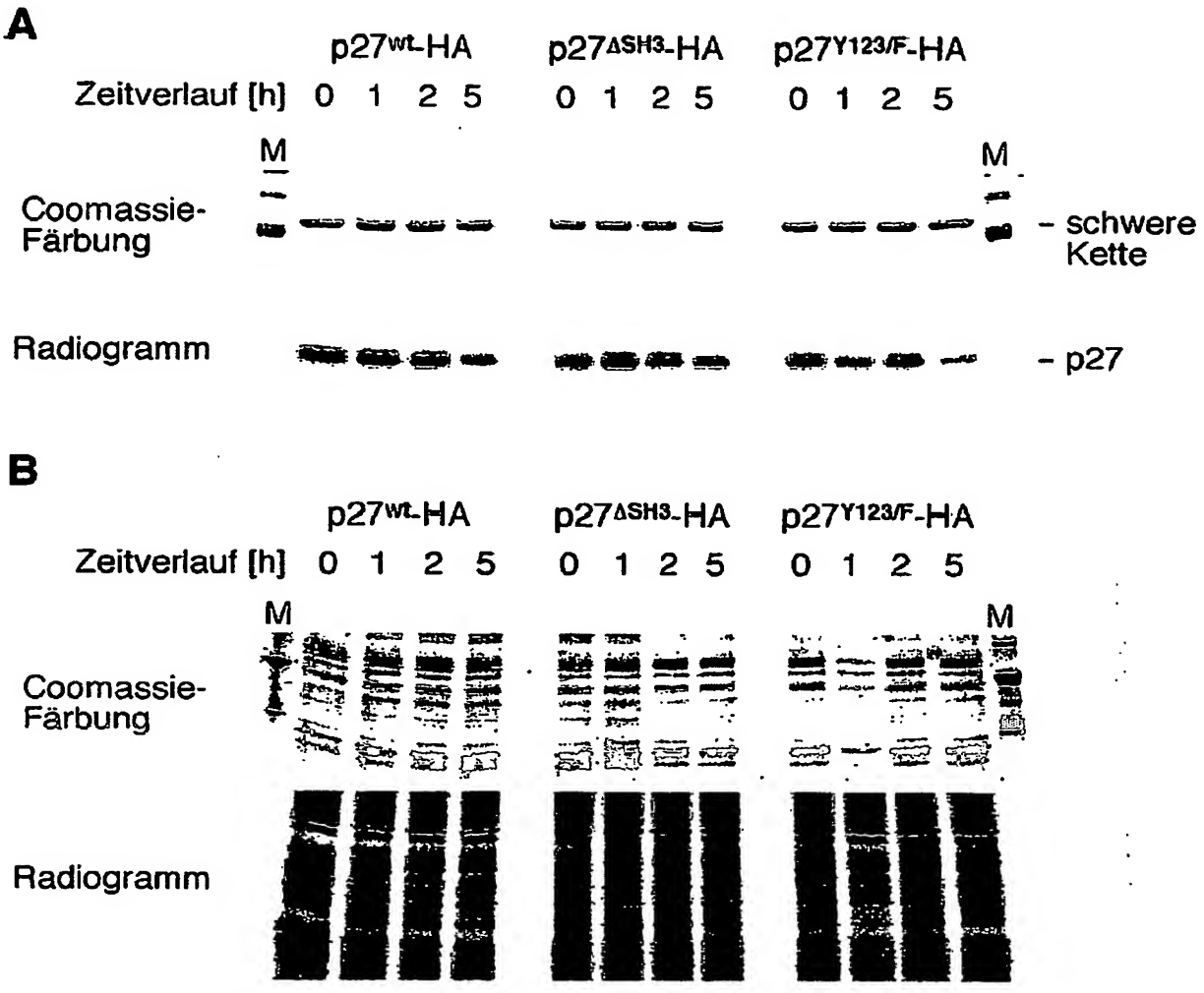
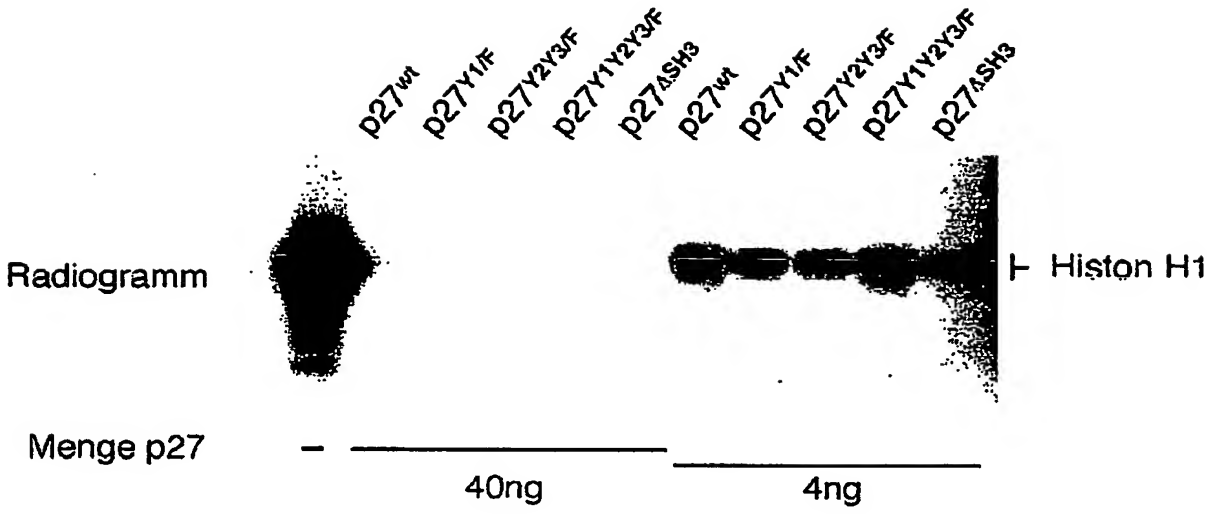

A
p27wt-HA
p27ΔSH3-HA
p27Y123/F-HA
Zeitverlauf [h] 0 1 2 5 0 1 2 5 0 1 2 5
M
M
Coomassie-Färbung
schwere Kette
Radiogramm
p27
B
p27wt-HA
p27ΔSH3-HA
p27Y123/F-HA
Zeitverlauf [h] 0 1 2 5 0 1 2 5 0 1 2 5
M
M
Coomassie-Färbung
Radiogramm

Fig. 4.29:


p27wt
p27Y1/F
p27Y2Y3/F
p27Y1Y2Y3/F
p27ΔSH3
p27wt
p27Y1/F
p27Y2Y3/F
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p27ΔSH3
Radiogramm
Histon H1
Menge p27
–
40ng
4ng

Fig. 4.30:

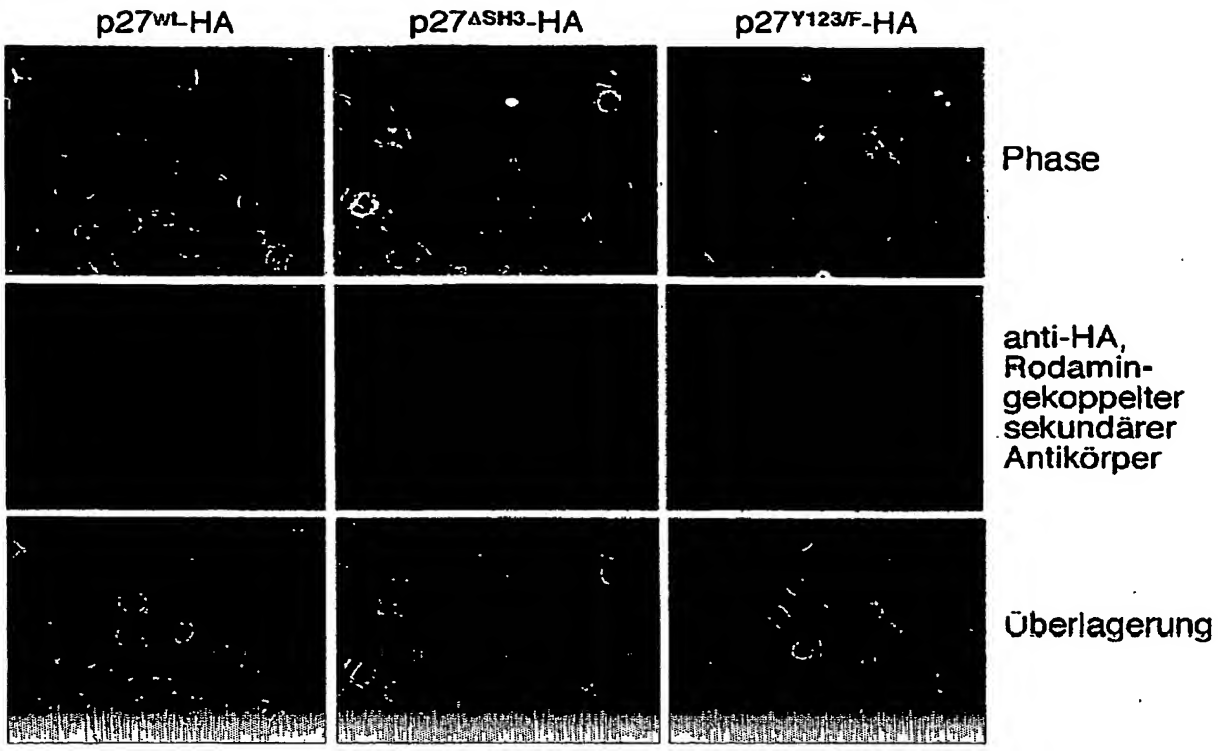

p27WT-HA
p27ΔSH3-HA
p27Y123/F-HA
Phase
anti-HA, Rodamin-gekoppelter sekundärer Antikörper
Überlagerung

Fig. 4.31:

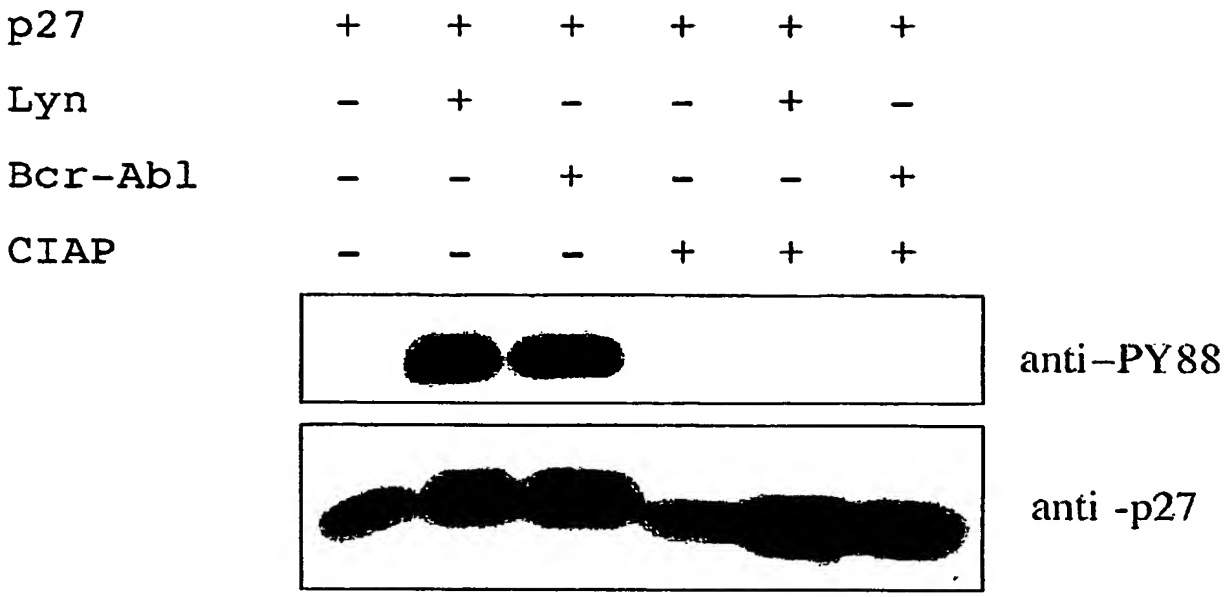
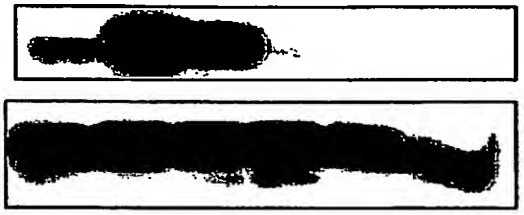


Fig. 4.32:

p27 wt	+	+	+			
p27 Y88,89F				+	+	+
Lyn		+			+	
Bcr-Abl			+			+



anti-pY 88

anti-p27

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35 40 45

Cys Arg Asp Met Glu Glu Ala Ser Gln Arg Lys Trp Asn Phe Asp Phe
50 55 60

Gln Asn His Lys Pro Leu Glu Gly Lys Tyr Glu Trp Gln Glu Val Glu
65 70 75 80

Lys Gly Ser Leu Pro Glu Phe Tyr Tyr Arg Pro Pro Arg Pro Pro Lys
85 90 95

Gly Ala Cys Lys Val Pro Ala Gln Glu Ser Gln Asp Val Ser Gly Ser
100 105 110

Arg Pro Ala Ala Pro Leu Ile Gly Ala Pro Ala Asn Ser Glu Asp Thr
115 120 125

His Leu Val Asp Pro Lys Thr Asp Pro Ser Asp Ser Gln Thr Gly Leu
130 135 140

Ala Glu Gln Cys Ala Gly Ile Arg Lys Arg Pro Ala Thr Asp Asp Ser
145 150 155 160

Ser Thr Gln Asn Lys Arg Ala Asn Arg Thr Glu Glu Asn Val Ser Asp
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gggccccggc gaggccggga tgagttggga ggaggcaggc ggcctggcac ctcacctgct 300

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35 40 45

Trp Asn Phe Asp Phe Val Thr Glu Thr Pro Leu Glu Gly Asp Phe Ala
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65 70 75 80

Gly Pro Arg Arg Gly Arg Asp Glu Leu Gly Gly Gly Arg Arg Pro Gly
85 90 95

Thr Ser Pro Ala Leu Leu Gln Gly Thr Ala Glu Glu Asp His Val Asp
100 105 110

Leu Ser Leu Ser Cys Thr Leu Val Pro Arg Ser Gly Glu Gln Ala Glu
115 120 125

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Leu Phe Gly Pro Val Asp His Glu Glu Leu Ser Arg Glu Leu Gln Ala
35 40 45

Arg Leu Ala Glu Leu Asn Ala Glu Asp Gln Asn Arg Trp Asp Tyr Asp
50 55 60

Phe Gln Gln Asp Met Pro Leu Arg Gly Pro Gly Arg Leu Gln Trp Thr
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Glu Val Asp Ser Asp Ser Val Pro Ala Phe Tyr Arg Glu Thr Val Gln
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Leu Glu Glu Ala Pro Glu Gln Leu Pro Ser Val Pro Val Pro Ala Pro
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Gln Leu His Ser Gly Ile Ser Gly Arg Pro Ala Ala Gly Thr Ala Ala
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Ala Ser Ala Asn Gly Ala Ala Ile Lys Lys Leu Ser Gly Pro Leu Ile
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Ser Asp Phe Phe Ala Lys Arg Lys Arg Ser Ala Pro Glu Lys Ser Ser
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